DOCTORAL THESIS

Hijacking of host cellular functions in Staphylococcus aureus pathogenesis

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Hijacking of host cellular functions in *Staphylococcus aureus* pathogenesis

by

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A mi familia y a Pablo, por estar siempre ahí

Abstract

Staphylococcus aureus is a facultative intracellular pathogen that invades and replicates within many types of human cells. *S. aureus* has shown to rapidly overcome traditional antibiotherapy by developing multidrug antibiotic resistance. Furthermore, intracellular *S. aureus* is protected from the last resort antibiotics – vancomycin, daptomycin and linezolid – since they are unable to achieve intracellular killing. Therefore, there is an urgent need to develop novel anti-infective therapies against *S. aureus*. Here, I proposed the exploitation of host-directed approaches as a promising strategy. Specifically, I employed three different approaches to unravel those host molecular factors and/or pathways hijacked by MRSA during intracellular infection.

By characterizing the host cell metabolism after MRSA infection, I observed several metabolic changes suggesting a starvation-induced autophagic flux and AMPK phosphorylation levels were also increased in MRSA-infected cells. It was hypothesized that intracellular *S. aureus* induces autophagy for energy generation and nutrient scavenging. Accordingly, host AMPK-inhibition halted *S. aureus* intracellular proliferation.

I further screened 140 host-directed drugs and found three host-directed tyrosine kinase inhibitors – Ibrutinib, Dasatinib and Crizotinib – that substantially impaired intracellular bacterial survival. Particularly, Ibrutinib increased host cell viability after *S. aureus* infection via inhibition of intracellular bacterial invasion or proliferation and we confirmed the importance of the host receptor EPHA2 for staphylococcal infection.

Lastly, I performed a genome-wide shRNA screen to test the importance of 16,000 host genes and identified several host genes important for intracellular MRSA infection. Specifically, I found that silencing the human gene *TRAM2* resulted in a significant reduction of intracellular MRSA. TRAM2 is associated with the endoplasmic reticulum SERCA pumps and accordingly, treatment with the SERCA-inhibitor Thapsigargin halted intracellular MRSA proliferation.

In summary, I identified three different host-directed drugs – Dorsomorphin, Ibrutinib and Thapsigargin – that impaired intracellular MRSA infection. These findings serve as an important example of feasibility for identifying host-directed therapeutics to tackle *S. aureus* host cell infection.

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Author´s declaration

I declare that the work included in this thesis is original, except where indicated by special reference in the text. The work was conducted by myself unless otherwise stated.

The thesis has not been presented to any University for examination, with the exception of work on the host-directed drug screening that was carried out together with Frederic Cooper towards his master's thesis, under my co-supervision.

Common abbreviations

AMPK	AMP-activated protein kinase
Agr	Accessory gene regulator
bp	Base pair
BTK	Bruton´s Tyrosine Kinase
CA-MRSA	Community Associated-MRSA
CFU	Colony Forming Units
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ER	Endoplasmic Reticulum
ERK	Extracellular signal–Regulated Kinases
EPHA2	Ephrin type-A Receptor 2
FBS	Foetal Bovine Serum
FnBP	Fibronectin Binding Protein
GC-MS	Gas Chromatography-Mass Spectrometry
GFP	Green Fluorescent Protein
HA-MRSA	Health Acquired-MRSA
HEK293	Human embryonic kidney cell line
IGC	Intracellular Growth Coefficient
kb	kilobases
KD	Knockdown
kDa	kiloDaltons
LB	Luria Bertani
LC-MS	Liquid Chromatography-Mass Spectrometry
µl	microlitre
µg	microgram
µM	micromolar
ml	millilitre
MOI	Multiplicity of Infection
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules

MSSA	Methicillin-Sensitive *Staphylococcus aureus*
NB	Nutrient Broth
NMR	Nuclear magnetic resonance
NWASP	Neuronal Wiskott–Aldrich Syndrome Protein
OD_{600}	Optical Density at 600 nm wavelength
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PSM	Phenol Soluble Modulins
PVL	Panton-Valentine Leukocidin
RNA	Ribonucleic acid
rpm	Revolutions per minute
SE	Standard Error
shRNA	Short hairpin RNA
SpA	Staphylococcal Protein A
SSC	Staphylococcal Cassette Chromosome
SSTI	Skin and Soft Tissue Infection
TBS	Tris Buffered saline
TCA	Tricarboxylic Acid cycle
TSS	Toxic Shock Syndrome
TSST-1	Toxic Shock Syndrome Toxin 1
V	Volt
WTA	Wall Teichoic Acids

1. Introduction

1.1. BIOLOGY OF *STAPHYLOCOCCUS AUREUS*

Staphylococci are Gram-positive cocci, with low G+C content of DNA, 1 µm in diameter, which tend to form clumps by growing in clusters, pairs and, occasionally, in short chains. *S. aureus* is characterized by its gold-pigmentation and for being catalase and coagulase positive, salt-tolerant and often haemolytic (Foster, T. 1996; Lowy 1998).

The genome of *S. aureus* consists of a single circular chromosome of approximately 2.8 Mbp, and extrachromosomal elements, such as conjugative and non-conjugative plasmids. Virulence and antibiotic resistance genes can be found in both the chromosome and extrachromosomal elements (Młynarczyk, Młynarczyk and Jeljaszewicz, 1998).

The cell wall of staphylococci species is composed of peptidoglycan, teichoic acids (WTA) and cell wall-associated proteins. Peptidoglycan is a sugar-based polymer, responsible for cell shape and bacterial integrity (Dmitriev *et al.*, 2004). Interestingly, the peptidoglycan of *S. aureus* possesses a unique pentaglycine side chain, which plays an important role in both pathogenicity and antibiotic resistance (Rohrer *et al.*, 1999). Furthermore, peptidoglycan may act as a Pathogen-associated molecular pattern

(PAMP), promoting the cytokine release by macrophages, complement activation as well as platelets aggregation (Kessler, Nussbaum and Tuazon, 1991). On the other hand, WTAs are covalently bound to the peptidoglycan layer (Lowy, 1998) and they are involved in nasal colonization (Weidenmaier *et al.*, 2004). Moreover, WTAs have also shown to increase staphylococcal virulence and to promote abscess formation by activating CD4+ cells through the Major Histocompatibility Complex II (MHC-II) (Wanner *et al.*, 2017).

In addition, most staphylococci strains produce microcapsules, which are formed by extracellular polysaccharides and enhance staphylococcal virulence by subverting phagocytosis from macrophages (Nilsson *et al.*, 1997; Kuipers *et al.*, 2016). Among the 11 different serotypes that have been identified, serotypes 5 and 8 are the most common in human infections caused by *S. aureus*. In particular, microcapsule type 5 is the most commonly identified in methicillin-resistant *S. aureus* strains (O´Riordan and Lee, 2004).

S. aureus is an opportunistic pathogen to humans, which are its natural reservoir. *S. aureus* is carried by approximately 30% of the global population (Sollid *et al.*, 2014; Sakr *et al.*, 2018). Humans can be colonized by both Methicillin-Sensitive and Methicillin-Resistant *S. aureus* strains (MSSA and MRSA, respectively) (Sollid *et al.*, 2014; Sakr *et al.*, 2018). Although the anterior nares of the nasal cavity is the most common carriage site of *S. aureus* (Foster, T. 1996; Wertheim et al. 2005), commensal *S. aureus* can also reside in axillae, vagina, pharynx and damaged skin surfaces (Lowy, 1998; Sollid *et al.*, 2014). Upon penetration of endothelial, epithelial or dermal barriers, due to a wound or tissue damage, the bacterium turns pathogenic. Several studies have shown that nasal colonization increase the risk of *S. aureus* infection by

10-fold (Davis *et al.*, 2004; Ellis *et al.*, 2004). Indeed, it has been traditionally believed that colonization precedes infection (Archer, 1998).

1.2. *S. AUREUS* INFECTIONS AND EPIDEMIOLOGY

S. aureus can cause a wide range of diseases, ranging from skin and soft tissue infections (SSTIs), deep tissue abscesses, endocarditis, osteomyelitis, toxic shock syndrome (TSS), pneumonia, bacteremia and sepsis (Lowy, 1998).

1.2.1. Skin and Soft Tissues Infections (SSTI)

Pathogenic *S. aureus* is able to cause a wide variety of SSTIs, from non-serious impetigo and uncomplicated cellulites to life-threatening infections, such as necrotizing fasciitis or pyomyositis (Tong et al. 2015). The importance of *S. aureus* in relation to SSTIs has increased in the past two decades due to the emergence of community-associated MRSA SSTIs (Otto, 2013). Specifically, the *S. aureus* USA300 strain has been identified as the most common isolate within SSTIs caused by Community-Associated MRSA (CA-MRSA) (King *et al.*, 2006).

1.2.2. Infective Endocarditis

Infective endocarditis starts with the damage of the cardiac endothelium, either by direct trauma, e.g. caused by an inflammatory response due to another disease. Afterwards, the exposure of subendothelial cells promote the production of proteins from the extracellular matrix and the deposition of fibrin and platelets, resulting in the

formation of vegetations. Infective endocarditis is the result of *S. aureus* colonization into these vegetations (Que and Moreillon, 2011). In industrialized countries, *S. aureus* is the most common causative agent of infective endocarditis (Witchitz *et al.*, 2005) and intravascular devices are commonly the source of the infection (A. Wang *et al.*, 2007). Moreover, other risk factors of *S. aureus* infective endocarditis are persistent bacteraemia, other healthcare-associated infections, as well as injection drug use (A. Wang *et al.*, 2007).

1.2.3. Osteomyelitis

Osteomyelitis is a bone infection that promotes inflammatory destruction, bone necrosis and new bone formation. It is quite common among children; however, the incidence of vertebral osteomyelitis in adults is increasing and the associated risk factors are advanced age, injection drug use, diabetes and immunosuppression (Mylona *et al.*, 2009; Akiyama *et al.*, 2013). Healthy bones are normally very resistant to infection. However, *S. aureus* has evolved several mechanisms to overcome this resistance and successfully colonize bone tissue. To achieve this, *S. aureus* expresses several surface proteins to gain adherence to the bone matrix and collagen (Cunningham, Cockayne and Humphreys, 1996). Besides, *S. aureus* is also able to invade osteoblasts and establish intracellular long-term infections (Shi and Xianlong, 2012; Kalinka *et al.*, 2014).

1.2.4. Toxic shock syndrome (TSS)

TSS caused by *S. aureus* is a superantigen-mediated illness, where the toxic shock syndrome toxin-1 (TSST-1) secreted by this pathogen is the main culprit (Musser *et*

al., 1990). TSST-1 cross-link T-cell receptor and Major Histocompatibility Class II Complexes (MHC-II) on antigen-presenting cells, leading to a large T-cell activation and cytokine release. Consequently, this causes an overactivation of the inflammatory response, followed by septic-shock and organ failure (Fraser *et al.*, 2000; Kalyan and Chow, 2008).

While TSS was thought to be associated with the use of tampons in menstruating women in the 1980s (Shands *et al.*, 1980), the incidence of menstrual and non-menstrual cases is very similar and SSTIs usually precedes non-menstrual TSS cases (DeVries *et al.*, 2011). In fact, non-menstrual toxic shock syndrome has been associated with higher mortality rates than menstrual toxic shock syndrome cases (Descloux *et al.*, 2008).

1.2.5. Pulmonary infections

S. aureus has also been identified as a common cause of pneumonia in both Community-Associated and Hospital-Acquired infections and both MSSA and MRSA strains are isolated from these patients (De la Calle *et al.*, 2016). It is also a common pathogen among cystic fibrosis patients (Schwerdt *et al.*, 2018). In the case of pneumonia caused by MSSA, the risk factors are the same as for general pneumonia: low body mass index, smoking, chronic lung and liver disease, history of pneumonia and diabetes. In the case of MRSA pneumonia, hospitalization, previous surgeries and long-term-care residence are included as risk factors (Klevens *et al.*, 2007).

Among the virulence factors of *S. aureus* to establish respiratory infections, the pore-forming α-toxin has been identified essential to develop clinical pneumonia (Bubeck Wardenburg *et al.*, 2007). At the cellular level, α-toxin is involved in the activation of

the NLRP3 inflammasome, which leads to necrotic lung injury (Kebaier *et al.*, 2012). Moreover, the increase of platelet-neutrophil aggregates – also activated by the α-toxin – contributes to tissue damage (Parimon *et al.*, 2013).

1.2.6. *S. aureus* bacteraemia

S. aureus bloodstream infections are linked to higher rates of mortality and morbidity than bacteraemia caused by other Gram-positive pathogens, such as *Streptococcus* or *Enterococcus* (Naber, 2009). The incidence of *S. aureus* bacteraemia is very high in the first year of life, low through the young adulthood and it gradually rises again with increasing age (van Hal et al. 2012; Tong et al. 2015). Males are also associated with a higher incidence than females, although the reasons behind this observation are still unclear (Humphreys, Fitzpatrick and Harvey, 2015).

Furthermore, there are several host-factors that increase the risk of developing *S. aureus* bacteraemia, including previous *S. aureus* infections or colonization, skin ulcers during hospitalization, the presence of venous catheters and surgical site infections, injection drug use, and an immunosuppressive condition (Naber, 2009). Additionally, haemodialysis patients also represent a risk group of invasive *S. aureus* infections, due to the presence of an intravascular device and the use of a tunnelled catheter for dialysis (FitzGerald *et al.*, 2011).

1.3. EMERGENCE OF METHICILLIN-RESISTANT *S. AUREUS* (MRSA)

Another important feature of *S. aureus* infection is the pathogen´s ability to acquire antibiotic resistance. Many infections are caused by strains that are resistant to multiple antibiotics. Similarly to other multidrug-resistant pathogens, MRSA infections are associated with higher costs and extended hospitalization periods, as well as higher morbidity and mortality rates (Ippolito *et al.*, 2010).

In 1959, methicillin was introduced to treat *S. aureus* infections caused by penicillin-resistant isolates. Two years later, in 1961, the first Methicillin-Resistant *S. aureus* isolate was reported in the UK (Jevons, 1961). Since then, several MRSA clones have evolved during the past decades by acquiring resistance to multiple antibiotics, that were introduced to treat MRSA infections (Hiramatsu *et al.*, 2001).

MRSA strains emerged by the acquisition of a large genetic element, known as the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (Katayama, Ito and Hiramatsu, 2000). SSC*mec* is a 21-67 kb fragment of DNA, which is integrated in the chromosome of MRSA near the origin of replication of *S. aureus*. This specific location may play a significant role since genes that are positioned close to the origin possess higher chances of being present at multiple copies after DNA replication and cell division. Moreover, this integration site is located in an Open Reading Frame (ORF) that, although its function is unknown, is well-conserved among clinical *S. aureus* isolates (Hiramatsu *et al.*, 2001).

Although the large size of SSC*mec* is comparable to a pathogenicity island, this chromosomal cassette does not contain any virulence genes (Ito, Katayama and Hiramatsu, 1999). It carries the *mecA* gene, which is responsible for β-lactam

antibiotics resistance (Katayama, Ito and Hiramatsu, 2000), together with the genes *mecl* and *mecR1*. *mecA* encodes the resistance to β-lactam antibiotics and *mecl* and *mecR1* act as regulators of the transcription of *mecA* (Zhang *et al.*, 2001). The β-lactam resistance in MRSA occurs via a specialised Penicillin-Binding Protein (PBP), which is a transpeptidase involved in the formation of cross-bridges in bacterial peptidoglycan. *mecA* encodes Penicillin-Binding Protein 2 (PBP2) with low binding affinity to β-lactam antibiotics (Hiramatsu *et al.*, 2001), which makes MRSA resistant to methicillin and all β-lactam antibiotics, including isoxazoyl penicillins (e.g., oxacillin) and broad spectrum β-lactam antibiotics, such as third-generation cephalosporins, cefamycins, and carbapenems (Chambers, 1997). Furthermore, the *SSCmec* cassette may also contain transposons and plasmids that carry resistance genes of non-β-lactam antibiotics (Hiramatsu *et al.*, 2001).

There are different types of SSC described and to date, 13 different types of SSCmec (I-XIII) have been identified for *S. aureus* (Baig *et al.*, 2018). Extensive dissemination of SSCmec has occurred among Staphylococci species; however, its origin remains unconfirmed (Hiramatsu *et al.*, 2001).

1.3.1. Healthcare-associated vs community-associated MRSA

Although MRSA infections were initially identified as nosocomial infections in the 1960s (Archer, 1998), the number of detected MRSA infection cases within healthy-community settings has risen in the past two decades, especially in the United States (Drews, Temte and Fox, 2006; David and Daum, 2010; Otto, 2013). The causative agents were classified as Community-Associated MRSA (CA-MRSA) and the first well documented case of CA-MRSA occurred among children between 1997 and 1999

in the United States (CDC, 1999). Although the origin of CA-MRSA is still unclear, some evidences point towards the acquisition of SSC*mec* type IV by the multiple community-based MSSA strains. The small size of SSC*mec* type IV makes feasible the easy horizontal transfer among different genetic backgrounds (Hiramatsu *et al.*, 2001).

Hospital-Acquired MRSA (HA-MRSA) infections are usually linked to advanced age, extended hospitalizations, intensive care unit stays, use of catheters and similar invasive procedures, haemodialysis patients, other clinical comorbidities and previous antibiotic exposure (Lowy, 1998). In contrast, community-associated MRSA (CA-MRSA) infections have been reported among young and healthy individuals without exposure to healthcare facilities or without carrying any of the associated risks to HA-MRSA (Drews, Temte and Fox, 2006). In paediatric cases, the dermatological condition is the underlying common factor associated with CA-MRSA infections; whereas in adults, the health of the skin, tobacco smoking and diabetes are the risk factors associated with this type of infections (Naimi *et al.*, 2003).

Regarding clinical manifestations, CA-MRSA infections are usually linked to SSTIs whereas HA-MRSA isolates are related to respiratory, urinary infections and bloodstream infections (Naimi *et al.*, 2003). However, when invading CA-MRSA infections such as necrotizing pneumonia occur, the patient´s prognosis is usually more negative (Drews, Temte and Fox, 2006).

CA-MRSA and HA-MRSA strains also differ in their genotypic and phenotypic characteristics. The resistance to β-lactam antibiotics and methicillin in HA-MRSA isolates is mainly acquired through SSC*mec* type II and III, which are relatively large cassettes and carry many other resistance determinants. By contrast, CA-MRSA strains carry SSC*mec* type IV, which is a smaller genetic element and contains *mecA*

as the only resistance gene (Ma *et al.*, 2002). Accordingly, CA-MRSA isolates are susceptible to most antimicrobials apart from β-lactam antibiotics and erythromycin, while HA-MRSA isolates are resistant to most available antibiotics (Naimi *et al.*, 2003). In addition, there are also differences in the expression of virulence factors among both strains. For example, Panton-Valentine Leucocidin (PVL) encoding genes are highly expressed in CA-MRSA strains (Watkins, David and Salata, 2012). PVL genes encode a pore-forming cytotoxin, which targets erythrocytes and leukocytes to induce an excessive inflammatory response, followed by tissue necrosis (Drews, Temte and Fox, 2006). This cytotoxin has been identified in 85% of CA-MRSA isolates causing necrotizing pneumonia, which may explain the higher mortality associated with this condition (Lina *et al.*, 1999).

1.4. PATHOGENESIS OF *S. AUREUS* INFECTIONS

S. aureus is a versatile pathogen, capable of invading and infecting a wide range of tissues, and such versatility is related to its diverse repertoire of virulence factors. These virulence determinants can mainly be divided into two categories: cell-surface-associated or secreted proteins (Fig. 1.1; (Gordon and Lowy, 2008)).

Expression of surface-associated proteins usually occurs during the exponential growth phase (replication), whereas secreted proteins are expressed during stationary phase. Hence, early expression of surface-associated proteins promotes initial tissue invasion and colonization, whereas the expression of secreted proteins, such as toxins, facilitates bacterial replication and *S. aureus* dissemination during infection (Gordon and Lowy, 2008) (Fig. 1.1).

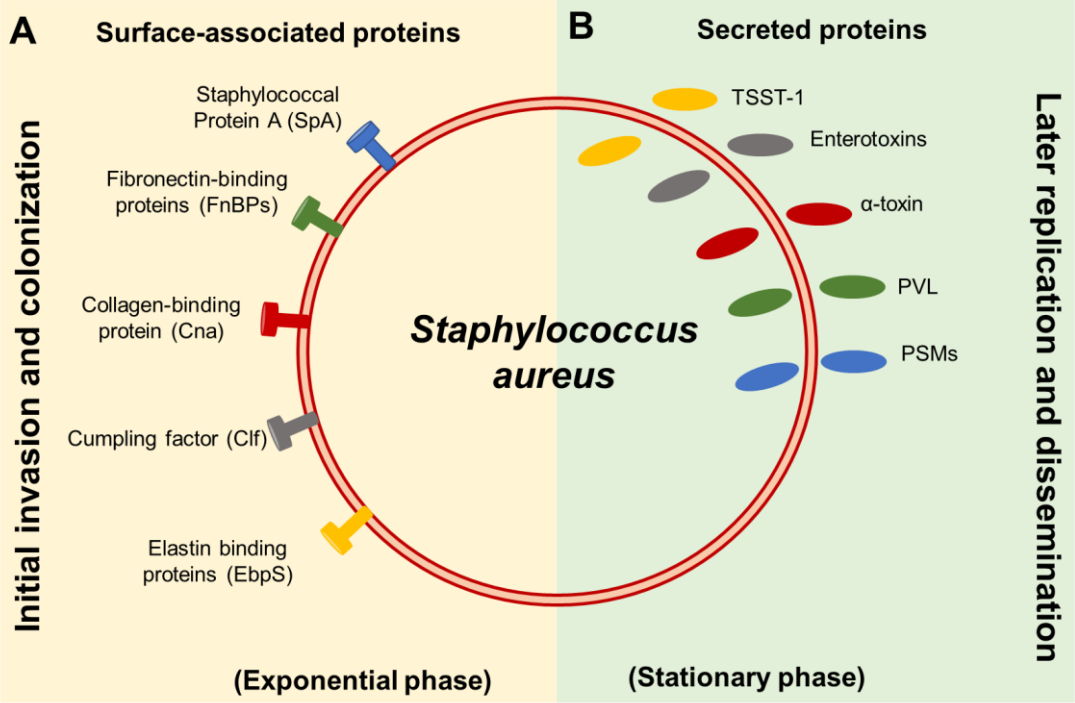

Figure 1.1. Surface and secreted proteins that act as virulence factors during *S. aureus* infections. (A) Surface-associated proteins are expressed during exponential growth phase and contribute to the initial host invasion and colonization. (B) Secreted proteins are expressed during stationary phase and are involved in *S. aureus* replication and dissemination. TSST-1, toxic shock syndrome toxin 1. PVL, Panton-Valentin Leukodicin. PSMs, Phenol-soluble modulins. Figure is adapted from Lowy, 1998.

1.4.1. Surface-associated proteins

S. aureus surface-associated virulence factors include capsular polysaccharides, staphyloxanthin and a group of proteins known as Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) (Foster and Höök, 1998).

Firstly, the role of capsules in *S. aureus* pathogenesis is mainly related to the prevention of phagocytosis by neutrophils, but it has also been associated with the enhancement of bacterial colonization and persistence on mucosal surfaces (O´Riordan and Lee, 2004).

Staphyloxanthin, the carotenoid pigment that confers the golden colour to *S. aureus* and it also confers protection to *S. aureus* against reactive oxygen species (ROS)

generated during oxidative burst of neutrophils. Accordingly, inhibition of staphyloxanthin synthesis makes *S. aureus* more vulnerable to the host-immune system, contributing to bacterial clearance in mice kidneys after intraperitoneal infection challenge (Song *et al.*, 2009).

Lastly, MSCRAMMs function as adhesin molecules to facilitate adherence to host-tissues (Foster and Höök, 1998; Gordon and Lowy, 2008). *S. aureus* is able to invade and colonize a wide range of tissues, including cartilage, bones, wounds or soft tissues and therefore, it takes advantage of different receptors to attach to specific tissues. For instance, bone colonization requires collagen-binding, whereas wound infection requires the expression of fibrinogen and fibronectin receptors. Among these structural proteins, the best well characterized are Staphylococcal protein A (SpA), fibronectin-binding proteins (FnBPs), collagen-binding protein (Cna), fibrinogen-binding protein (clumping factor) and elastin binding proteins (EbpS) (Fig. 1.1).

1.4.1.1. Staphylococcal protein A (SpA)

SpA is a 42 kDa protein, covalently anchored in the staphylococcal cell wall. It possesses five immunoglobulins-binding domains and each unit is capable of binding to the Fc region of different mammalian IgG subclasses (Sjödahl, 1977; Moks *et al.*, 1986), which has been directly associated with the protection of *S. aureus* from opsonophagocytosis (Peterson *et al.*, 1977). In addition, it can also interact with the Fab domain of B-cell receptors (Sasso, Silverman and Mannik, 1989; Jansson, Uhlén and Nygren, 1998) and such interaction promotes B-cells activation (Goodyear and Silverman, 2003). Overall, protein A has shown the potential to alter the host-immune response against *S. aureus* during infection and therefore, it has been recently considered as a novel vaccine antigen (Kobayashi and Deleo, 2013). Moreover, high

levels of protein A have been associated to *S. aureus* nasal carriage (Muthukrishnan *et al.*, 2011).

1.4.1.2. Fibronectin-binding proteins (FnBPs)

S. aureus has two main fibronectin-binding proteins, FnBPA and FnBPB, whose molecular weight is approximately 200 kDa and they are only expressed during early exponential growth phase (Shinji *et al.*, 2011). The structure of both FnBPA and FnBPB is quite similar, resulting in high sequence homology (68%) (Meenan *et al.*, 2007), which suggests similar functions in *S. aureus* virulence.

Both FnBPA and FnBPB are major determinants during *S. aureus* invasion and internalization into non-professional phagocytes, such as endothelial and epithelial cells, keratinocytes and fibroblasts. Internalization into non-phagocytic cells occurs by a "zipper-type mechanism", which involves the binding of FnBPA and FnBPB to the host membrane α5β-integrin via a fibronectin bridge (Sinha *et al.*, 1999; Edwards *et al.*, 2011; Shinji *et al.*, 2011). Accordingly, deletion of FnBPA reduced *S. aureus* invasion in human embryonic cells (HEK293T) by 80-85%, while a double knockout of FnBPA and FnBPB resulted in a decrease of 95%. These results suggest that although both FnBPs proteins are important during *S. aureus* internalization, FnBPA may play a more critical role (Sinha *et al.*, 1999).

Due to its important role during *S. aureus* internalization, FnBPs are described as a major virulence factor of *S. aureus* pathogenesis. This importance is further reflected by the high expression of FnBPs found among most clinical isolates (around 70%) (Hauck and Ohlsen, 2006; Shinji *et al.*, 2011).

1.4.1.3. Collagen-binding protein (Cna)

The collagen-binding protein Cna is a cell-anchored protein, expressed by *S. aureus* to attach to collagen-rich tissues, such as cartilage, which is particularly rich in collagen II (Switalski *et al.*, 1993). Collagen, the most abundant protein in humans, is responsible for the structural support of tissues and therefore, it represents an important ligand for the initiation and persistence of numerous bacterial infections (Madani, Garakani and Mofrad, 2017).

Moreover, attachment of *S. aureus* to cartilage and bone tissue is directly correlated with the expression of Cna, showing its involvement in the development of septic arthritis (Switalski *et al.*, 1993; Patti *et al.*, 1994). It has also been described as a major virulence factor in the pathogenesis of osteomyelitis, keratitis and endocarditis (Hienz *et al.*, 1996; Rhem *et al.*, 2000; Elasri *et al.*, 2002).

1.4.1.4. Clumping factors (Clfs)

Fibrin is the major constituent of blood clots and fibrinogen/fibrin is one of the predominant proteins found in implanted materials. The ability of *S. aureus* to adhere to surfaces coated with these two proteins is crucial to stablish implanted-associated infections (Mcdevitt *et al.*, 1997).

Clumping factor A has the ability to adhere to both fibrinogen and fibrin and it is also responsible for bacterial cells clumping (McDevitt *et al.*, 1994). Specifically, ClfA has two binding sites of within the γ-chain of the fibrinogen (Mcdevitt *et al.*, 1997; Geoghegan *et al.*, 2010). Moreover, ClfA is also associated with platelet aggregation, which are thought to promote endovascular infection and dissemination of *S. aureus* by exposing the damaged endothelium to the pathogen (Niemann *et al.*, 2004).

Clumping factor B is a protein able to adhere to fibrinogen. Although its general organisation is quite similar to ClfA, the binding mechanism of these two proteins is different: ClfB binds the α and β-chains of fibrinogen, whereas ClfA exclusively recognize γ-chains (Eidhin *et al.*, 1998). More recent studies revealed that ClfB is also able to bind cytokeratin 10, promoting the adhesion to squamous epithelial cells, which may be important for nasal colonization (O'Brien *et al.*, 2002; Walsh *et al.*, 2004).

1.4.1.5. Elastin binding protein (EbpS)

EbpS is a surface-associated protein with the ability to bind soluble elastin as well as the tropoelastin precursor (Park *et al.*, 1991; Nakakido, Tanaka and Tsumoto, 2007). Elastin is one of the major constituents of the elastic fibres within the extracellular matrix, which plays an important role in maintaining the structural integrity in those tissues where reversible extensibility is required (Park *et al.*, 1991). *S. aureus* is capable to infect human tissues that are rich in elastin, such as lungs and skin and therefore, elastin-binding proteins (EbpS) may facilitate the invasion and colonization of these tissues (Downer *et al.*, 2002).

1.4.2. Secreted proteins

Although research has been traditionally focused on the study of cell surface-associated proteins in *S. aureus* pathogenesis, the relevance of secreted-proteins in the initiation and progression of staphylococcal infections is now becoming clear. Many are identified as toxins, and their distinction from other virulence determinants is due to their potential to interfere directly with the host (Otto, 2014). These toxins play key roles in subverting the host-immune system by disrupting host cells and tissues, as

well as interfering with the host-immune responses to release nutrients that facilitate bacterial replication (Lin and Peterson, 2010). Current antibiotics to treat *S. aureus* infections, kill the bacterium by lysing the staphylococcal cell wall or by inhibiting its biosynthesis. However, these drugs are unable to inhibit *S. aureus* toxins production or to neutralize their effects on host cells (Stevens *et al.*, 2007).

Among the plethora of staphylococcal secreted virulence factors, the best well-characterized proteins can be classified into two different categories: superantigens and pore-forming toxins.

1.4.2.1. Superantigens

Enterotoxins and the Toxic Shock Syndrome toxin 1 (TSST-1) are included in this category. More than 20 superantigens have been identified among different *S. aureus* strains and approximately 60% of clinical isolates contain at least one superantigen (Becker *et al.*, 2003), showing its relevance in *S. aureus* infections.

The main function of superantigens is to disturb the host immune response in order to ensure bacterial survival and persistence in the host. These toxins are able to activate T-cells and antigen-presenting cells – such as macrophages and dendritic cells – leading to a massive release of cytokines and chemokines (Bernal *et al.*, 1999; Fraser *et al.*, 2000). This cytokine storm causes the symptoms that are observed in the TSS: fever, rash, desquamation of the palms of hands and feet, hypotension and multiorgan failure (DeVries *et al.*, 2011). To promote *S. aureus* pathogenesis, superantigens might also have direct effects on specific cells. For example, TSST-1 has pro-inflammatory and cytotoxic effects on endothelial cells, which leads to capillary leakage and TSS symptoms (Lee *et al.*, 1991). Moreover, TSST-1 also acts as a proinflammatory toxin

in epithelial cells, stimulating neutrophil recruitment, which leads to subsequent tissue destruction (Peterson *et al.*, 2005).

On the other hand, bacteria expressing enterotoxins (SEs) are capable to induce emesis and diarrhoea when ingested, caused by an SE-induced inflammation in the abdominal area (Dinges *et al.*, 2000). *S. aureus* produce at least 5 enterotoxins: SEA, SEB, SEC, SED and SEE. In particular, Staphylococcal enterotoxin B (SEB) has been described as a potential biological weapon (Zapor and Fishbain, 2004), whereas Staphylococcal enterotoxin C (SEC) is associated with CA-MRSA in invasive endocarditis and sepsis (Salgado-Pabón *et al.*, 2013).

1.4.2.2. Pore-forming toxins

S. aureus secretes a great number of pore-forming toxins, including: cytolysins (α-toxin), toxins from the leucocidin family (PVL), and phenol-soluble modulins (PSMs) (Fig.1.1). They differ from each other in the structure and target specificity, despite their function on host cells is very similar. They create pores in host cell membranes, causing leakage and inflammation at low doses and cell lysis at higher doses (Lin and Peterson, 2010).

The most well-characterized cytolysin is the α-toxin (Berube and Wardenburg, 2013). It is encoded by the gene *hla*, which is expressed in all clinical isolates (Dinges *et al.*, 2000). The α-toxin is cytolytic to a wide range of mammalian cells, such as erythrocytes, leukocytes (monocytes and macrophages), platelets, fibroblasts and epithelial cells (Berube and Wardenburg, 2013). The role of the α-toxin in staphylococcal pneumonia has also been demonstrated in mice, where the mortality rate was associated with the concentration of secreted α-toxin by *S. aureus* (Kebaier *et al.*, 2012). At lower concentrations, the α-toxin is dependent on the disintegrin and

metalloproteinase ADAM10, leading to the disruption of focal adhesions (Wilke and Wardenburg, 2010; Inoshima *et al.*, 2011).

Another member of pore-forming toxins is the Panton-Valentin Leukodicin (PVL), a virulence determinant associated with CA-MRSA strains. PVL is a bicomponent toxin, encoded by *lukS* and *lukF* genes (Otto 2010; Watkins et al. 2012; Otto 2013). PVL forms pores in the membrane of neutrophils – a major component in the host-immune response against bacterial infections – causing their lysis. PVL was firstly described by Panton and Valentine in 1932 and associated with SSTIs; however, its virulence in *S. aureus* infections remains controversial. For example, Voyich and co-workers showed that CA-MRSA strains lacking PVL were as virulent as those strains expressing the toxin in mice sepsis and abscess formation (Voyich *et al.*, 2006). Another study compared two CA-MRSA strains (*S. aureus* USA300 and *S. aureus* USA400) in a mouse pneumonia model and reported that it was α-toxin, and not PVL, the responsible for mice mortality (Bubeck Wardenburg *et al.*, 2007). However, Löffler et al. (2010) investigated the cytotoxic effect of PVL in neutrophils from human, mice, rabbit and monkeys and they showed that murine neutrophils are not sensitive to PVL, suggesting that mice models do not fully replicate staphylococcal human infections. In contrast, rabbit neutrophils were much more susceptible to PVL than murine cells, indicating a closer model to human disease (Löffler *et al.*, 2010). Collectively, PVL is certainly a virulence factor in CA-MRSA infections but its contribution to the severity of the disease seems to occur in a strain and host-specific manner.

Lastly, *S. aureus* is also capable of producing high amounts of small and amphipathic peptides with detergent-like properties, which are known as Phenol-Soluble Modulins (PSMs). Production of PSMs *in vitro* is higher in CA-MRSA than HA-MRSA isolates

(R. Wang *et al.*, 2007). The main role of PSMs on *S. aureus* pathogenesis is the leukocytes' destruction and thus, these toxins play a crucial role in the host-immune evasion of *S. aureus* (R. Wang *et al.*, 2007; Otto, 2014).

In addition to superantigens and pore-forming toxins, *S. aureus* also secretes several extracellular enzymes and other proteins, which are involved in host-tissue disruption, modulation of host immune responses and/or inactivation of host antimicrobial mechanisms (Lin and Peterson, 2010). Some of these secreted factors are briefly summarized in table 1.1.

Name	Putative function	Reference
Exoenzymes		
Serine proteases	Inactivate neutrophils activity	(Lin and Peterson, 2010)
Cysteine proteases (Staphopain)	Block of neutrophil activation	(Laarman *et al.*, 2012)
Aureolysin	Inactivate antimicrobial peptides	(Sieprawska-Lupa *et al.*, 2004)
Staphylokinase (SAK)	Activate plasminogen; Inactivate antimicrobial peptides	(Bokarewa, Jin and Tarkowski, 2006)
Miscellaneous proteins		
Staphylococcal Complement Inhibitor (SCIN)	Inhibit complement activation	(Rooijakkers *et al.*, 2005)
Chemotaxis Inhibitory Protein of *S. aureus* (CHIPS)	Inhibit chemotaxis	(Foster, 2005)
Extracellular Adherence Protein (Eap)	Inhibit neutrophil migration	(Harraghy *et al.*, 2003)

Table 1.1. Extracellular enzymes and other proteins secreted by *S. aureus* during infection.

1.5. REGULATION OF *S. AUREUS* VIRULENCE FACTORS DURING INFECTION

As aforesaid, the process of *S. aureus* infection can be divided in two separate stages: (i) an adhesion/invasion phase, and (ii) a proliferation phase, which are characterized by the expression of different virulence factors (surface-associated vs secreted, respectively). The transition of these two phases and the subsequent expression of different virulence factors are commanded by a complex regulatory network, including the quorum-sensing Accessory gene regulator (Agr) system, the transcriptional regulators of the Sar family, the two-component regulatory systems ArlRS and SaeRS, and the sigma factor B (σB) (Hauck and Ohlsen, 2006; Wang and Muir, 2016).

The Agr quorum-sensing system is a 3.5 kb locus that comprises two transcriptional units (RNAII and RNA III), whose transcription is controlled by two promoters (Promoter 2 and Promoter 3). The RNAII gene cluster encodes a DNA-binding response regulator (AgrA) and a sensor kinase (AgrC). Additionally, the precursor of Auto-Inducing Peptide (AIP) – AgrD – is firstly processed by membrane-bound peptidase – AgrB – to generate an intermediate (Wang and Muir, 2016). AgrC senses the concentration of the AIP and at high concentrations of AIP, AgrC phosphorylates AgrA. Consequently, phosphorylated AgrA enhances *agr* transcription by binding to the promoter 2 region of RNA II and the promoter 3 region of RNA III (Le and Otto, 2015; Horn *et al.*, 2017). Besides AIP, the Agr system can also be activated by other regulators, such as SarA and SrrAB (Heinrichs, Bayer and Cheung, 1996; Yarwood, McCormick and Schlievert, 2001), or by environmental conditions, such as pH or glucose levels via *codY* (Regassa, Novick and Betley, 1992).

RNA III is considered the main intracellular effector of the Agr system. RNA III inhibits the expression of surface-associated proteins, allowing the upregulation of secreted toxins (Fechter *et al.*, 2014). This regulatory mechanism reflects the temporal requirements for different virulence factors during *S. aureus* infection, which is directly related to bacterial cell density and *agr*-regulation. At the beginning of the infection, low bacterial density – and low expression of *agr* – promote the expression of surface proteins required for initial adhesion. Once infection is established, higher bacterial density leads to the upregulation of the Agr system and the subsequent production of toxins (Le and Otto, 2015). Moreover, activation of the Agr system can be triggered within host cells.

Nevertheless, the quorum-sensing Agr system upregulates 174 genes, and 90 of those genes are regulated in a RNAIII-independent manner. Among the virulence factors directly-controlled by the *Agr* system, the staphylococcal protein A (SpA), α-toxin (Hla), bicomponent toxins (PVL, lukSF, lukDE) and proteases (e.g. staphopain) are included (Queck *et al.*, 2008).

In summary, the quorum-sensing Agr system plays an essential role in *S. aureus* pathogenesis. The importance of the upregulation of virulence factors controlled by the Agr system in acute infection has been shown in different animal models, including SSTIs, infective endocarditis, pneumonia, septic arthritis and osteomyelitis (Le and Otto, 2015). Accordingly, *agr* deletion mutants showed a reduced virulence in a wide range of infections (Horn *et al.*, 2017), which makes the Agr system a potential drug target for anti-staphylococcal therapies. However, silencing the Agr system activates *S. aureus* biofilm formation, which may support the persistence of chronic infections (Kong, Vuong and Otto, 2006).

In addition to the well-characterized Agr quorum-sensing system, *S. aureus* further possess other regulatory components, important for *S. aureus* pathogenesis. The two-component ArlRS regulates *S. aureus* exotoxin production and induces the expression of the DNA-binding protein SarA (Fournier, Klier and Rapoport, 2001). Also, ArlRS enhance intracellular *S. aureus* growth in the presence of calprotectin, which is an immune protein mainly expressed by neutrophils.

Lastly, the sigma factor B (σB) also contributes to *S. aureus* pathogenicity. σB is required for tolerance to low pH and hydrogen peroxide, which is associated with *S. aureus* survival after being ingested by phagocytes (Chan *et al.*, 1998). In addition, the σB-activating protein RsbU is needed for the internalization and intracellular growth of *S. aureus* within HUVEC and THP-1 cells (Olivier *et al.*, 2009). Furthermore, σB has shown to promote intracellular persistence through the formation of SCVs, being crucial in chronic infections (Tuchscherr *et al.*, 2015).

1.6. BIOFILM FORMATION

S. aureus is also characterized by its ability to produce biofilms, enhancing bacterial survival and persistence in host-tissues. A biofilm is a microbial community whose cells are attached to a surface or to other cells. Bacterial growth within biofilms might be important during infection since it provides a niche, where the pathogen is protected from several clearance mechanisms. Biofilms can prevent the access of certain immune cells, such as macrophages (Scherr *et al.*, 2014) and they also exhibit increased antibiotic resistance (de la Fuente-Núñez *et al.*, 2013).

The process of biofilm formation can be subdivided in three stages: (i) initial attachment, (ii) biofilm maturation and (iii) dispersal (Lister and Horswill, 2014).

(i) *S. aureus* mediates initial attachment by expressing surface-associated proteins (MSCRAMMs). Particularly, protein A, fibronectin-binding proteins (FnBPs), *S. aureus* surface protein (SasG), biofilm-associated protein (Bap) and clumping factor B (ClfB) are important for both initial bacterial attachment and biofilm accumulation (Foster *et al.*, 2014; Lister and Horswill, 2014).

(ii) Afterwards, biofilm maturation is characterized by bacterial cell division and the formation of an extracellular polymeric matrix. The composition of this extracellular matrix is highly strain-dependent (Artini *et al.*, 2012); however, host-factors, polysaccharides, extracellular DNA (eDNA), and secreted and lysis-derived proteins are commonly found within its composition (Montanaro *et al.*, 2011; Foster *et al.*, 2014). Another major biofilm component is the Polysaccharide Intracellular Adhesin (PIA), which is equally important in both *S. aureus* and *S. epidermis* biofilms, and confers structural integrity to biofilms *in vitro* and *in vivo* (Lister and Horswill, 2014).

(iii) Eventually, individual bacterial cells are able to disperse from the original biofilm site, establishing new loci of infection (Boles and Horswill, 2011). Among the staphylococcal factors that support biofilm dispersal, PSMs have shown to play an important role. They are able to disrupt non-covalent forces that maintain the extracellular matrix together, creating channels that are essential for the dissemination of bacterial cells to new sites (Peschel and Otto, 2013; Le *et al.*, 2014). In addition, the induction of Agr complex mediates biofilm dispersal by triggering proteases production (Boles and Horswill, 2008).

Taking altogether, persistence of *S. aureus* in chronic infections is highly associated with biofilm formation and bacterial growth within it. In particular, *S. aureus* cause osteomyelitis and endocarditis by persisting on host tissues like bones or heart valves, respectively (Parsek and Singh, 2003). It can also adhere to implanted materials – such as catheters, prosthetic joints and pacemakers – that become coated with host-protein, facilitating bacterial attachment through matrix-binding proteins (François *et al.*, 1996; Barrett and Atkins, 2014).

1.7. INTRACELLULAR FATE OF *STAPHYLOCOCCUS AUREUS*

While traditionally *S. aureus* was exclusively classified as an extracellular pathogen (Finlay and Cossart, 1997), it has been shown that this pathogen is also able to subvert phagocytosis by macrophages and neutrophils and to be internalized by non-phagocytic cells, such as epithelial and endothelial cells, osteoblasts, fibroblasts and keratinocytes (Garzoni and Kelley, 2009; Fraunholz and Sinha, 2012; Horn *et al.*, 2017). It has even been shown to survive within neutrophils and human monocyte-derived macrophages (Gresham *et al.*, 2000; Kubica *et al.*, 2008). Therefore, and taking all these evidences together, *S. aureus* is currently considered a facultative intracellular pathogen.

Intracellular survival strategies of *S. aureus* are very diverse, depending on the bacterial strain as well as the type of infected host cell (Strobel *et al.*, 2016). Particularly, most of the host-pathogen interactions occurring during *S. aureus* infection could be classified into four categories: internalization into host cells, intracellular persistence, phagosomal escape and host-cell death induced by *S. aureus*.

1.7.1. *S. aureus* internalization into non-phagocytic cells

Mechanistically, invasion of non-professional phagocytes by *S. aureus* is achieved via a zipper-type mechanism, which is mainly mediated by the interaction between *S. aureus* FnBPA and FnBPB, fibronectin and the $\alpha_5\beta_1$-Integrins of the host cell (Fig. 1.2A) (Sinha and Fraunholz, 2010; Fraunholz and Sinha, 2012; Foster, 2016). The efficiency of this type of adhesion (FnBPs / Fibronectin / $\alpha_5\beta_1$-integrins pathway) differs between different cell lines. For example, invasion of endothelial cells requires only one high-affinity Fibronectin Binding Receptor (FnBR) (Edwards *et al.*, 2010), whereas three FnBRs are required to get internalized in keratinocytes (Edwards *et al.*, 2011).

Moreover, epithelial or endothelial cells usually do not expose $\alpha_5\beta_1$-integrins in their surfaces to be accessible by the pathogen. Only sub-confluent cell monolayers or damaged tissues express these fibronectin receptors, which may illustrate why intracellular *S. aureus* is commonly found in disrupted tissues (Horn *et al.*, 2017). Nevertheless, *S. aureus* can also lead to tissue destruction by α-toxin production, which ability to disrupt the barrier function of epithelial cells is mediated by interfering with tight junction integrity (Kwak *et al.*, 2012). Interestingly, α-toxin has further shown to increase β_1-Integrins in mast cells, promoting *S. aureus* internalization (Goldmann *et al.*, 2015). In line with this latter observation, cells lacking β_1-integrins do not internalize *S. aureus* at the same rate as cells expressing these integrins (Hauck and Ohlsen, 2006). Furthermore, it has been recently demonstrated that β_1-integrins are ectopically expressed on the surface of epithelial cells in cystic fibrosis (CF) patients, which explains the persistent *S. aureus* colonization in the lungs of CF patients (Li *et al.*, 2017).

In summary, interaction between bacterial FnBPs and host integrins plays an important role during *S. aureus* internalization in non-phagocytic cells. This interaction further leads to signal transductions in the host cell to promote cytoskeleton rearrangements, which are also necessary for the successful internalization of *S. aureus* into the host cell (Dziewanowska *et al.*, 1999). The relevance of host-actin cytoskeleton is in concordance with previous studies where treatment of cells with cytochalasin D – an inhibitor of actin polymerization – resulted in a significant reduction of *S. aureus* internalization (Sinha *et al.*, 1999). Specifically, the signalling pathway involves focal adhesion kinases (FAKs) and the activation of Src, which consequently recruit cortactin to facilitate host-actin polymerization (Fig. 1.2) (Agerer *et al.*, 2005). Some specificity among cell lines is also observed regarding the intracellular signalling cascades activated during pathogen internalization. For instance, the activation of phosphoinositide-3-kinase (PI3K)-Akt pathway is important during *S. aureus* internalization by endothelial cells (Oviedo-Boyso *et al.*, 2011). On the other hand, the Extracellular Signal-Related Kinases (ERK) and c-JUN kinases (JNK) are involved in the internalization of *S. aureus* inside osteoblasts, whereas the Mitogen-Activated Protein Kinase p38 (MAPK) is also upregulated along with ERK in Hep-2 cells (Ellington *et al.*, 2001).

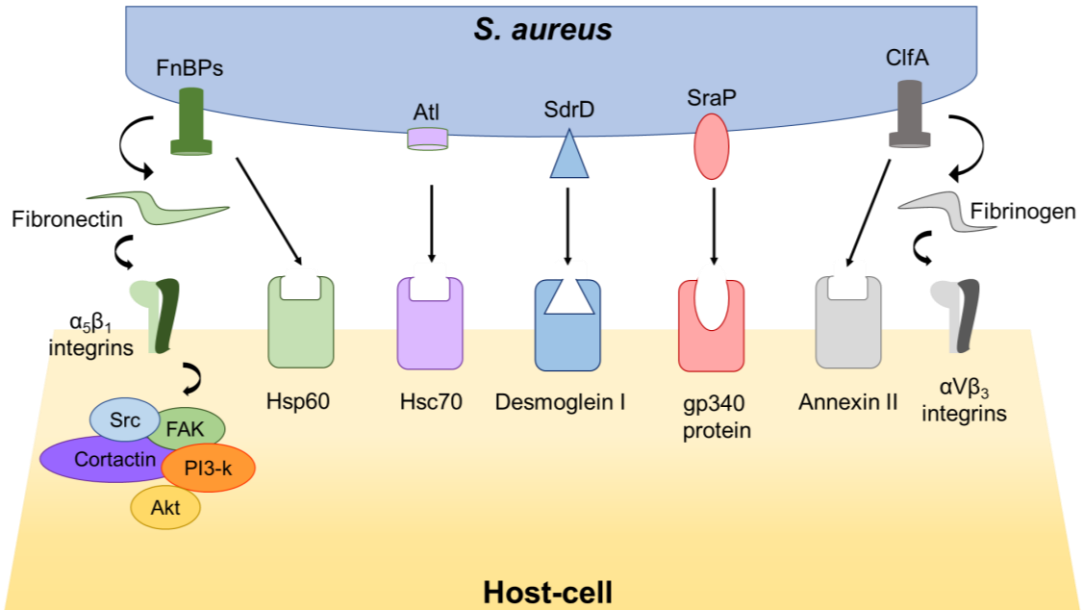

Figure 1.2. *S. aureus* mechanisms of internalization into host-cells. FnBPs, Fibronectin-binding proteins. Atl, autolysin. SdrD, Serine Aspartate Repeat-containing protein D. SraP, Serine-Rich Adhesin for platelets. ClfA, Clumping factor A. FAK, Focal Adhesion Kinase. PI3-k, phosphoinositide-3-kinase. Hsp60, Heat Shock Protein 60. Hsc70, heat shock cognate protein 70. Figure adapted from Josse et al. 2017.

Besides the main internalization pathway (FnBPs / Fibronectin / $\alpha_5\beta_1$-integrins), FnBPs are also able to directly bind to the Human Heat Shock Protein 60 (Hsp60), which is exposed in the surface of epithelial cells, in order to promote internalization (Dziewanowska *et al.*, 2000). Additionally, there are other bacterial factors that also interact with specific host factors to facilitate host cell invasion and internalization of *S. aureus*. Among these secondary fibronectin-independent mechanisms, the most common bacterial factors are Autolysin (Atl), Clumping Factor A, Serine Aspartate Repeat-containing protein D (SdrD) and Serine-Rich Adhesin for platelets (SraP), which bind different host-receptors (Fig 1.2). For example, the staphylococcal Atl protein acts as an adhesin by directly binding to the heat shock cognate protein 70 (Hsc70) (Hirschhausen *et al.*, 2010); SdrD mediates adherence to keratinocytes through Desmoglein 1 binding (Askarian *et al.*, 2016); and SraP binds the scavenger protein gp340 to promote internalization into A549 cells (Yang *et al.*, 2014). On the

other hand, the mechanism of ClfA differs depending on the cell line. In epithelial cells, ClfA directly binds to Annexin 2 (Ashraf, Cheng and Zhao, 2017), whereas in endothelial cells ClfA binds to αVβ3-integrin using fibrinogen as a bridge (McDonnell *et al.*, 2016). Moreover, it has been shown that *S. aureus* Newman strain mediates internalization to fibroblasts and epithelial cells *via* Eap (Hussain *et al.*, 2002), although the molecular mechanism behind this internalization pathway is still not fully elucidated (Fig. 1.2).

1.7.2. Intracellular persistence, the role of Small Colony Variants (SCVs)

The efficiency of intracellular *S. aureus* survival depends on several factors: (i) the bacterial strain, (ii) the host-cell and its gene expression, (iii) the multiplicity of infection (MOI), and (iv) the bacterial growth phase employed for the infection (Fraunholz and Sinha, 2012). However, the intracellular survival of *S. aureus* for extended periods of time has been reported into different mammalian cell lines (Garzoni and Kelley, 2009; Fraunholz and Sinha, 2012; Horn *et al.*, 2017). In particular, *S. aureus* can survive and replicate inside phagocytic cells, such as neutrophils and macrophages (Gresham *et al.*, 2000) and interestingly, internalized *S. aureus* in macrophages express anti-apoptotic factors in order to prolong bacterial survival (Koziel *et al.*, 2009). *S. aureus* can also survive inside migratory phagocytes, facilitating its own dissemination to new points of infection (Horn *et al.*, 2017).

In general, intracellular persistence has been commonly associated with small colony variants (SCVs) forms, which have further been correlated with chronic and recurrent *S. aureus* infections (Kahl, 2014). SCVs are characterized by a reduced metabolic

activity, slow growth rates and increased antibiotic resistance (Proctor *et al.*, 2006; Sendi and Proctor, 2009). The visual features of SCV that distinguish them from the wild-type phenotype include small colony size, reduced golden pigmentation, and decreased haemolysis on blood agar plates (Sendi and Proctor, 2009; Kahl, 2014). At molecular level, there are also some differences between both phenotypes. For instance, the gene *sigB* is up-regulated in SCV forms (Horsburgh *et al.*, 2002) and this up-regulation promotes the expression of Clf and FnBPs, making SCVs well prepared for the attachment to host-cells (Vaudaux *et al.*, 2002).

Once *S. aureus* has been internalized into eukaryotic cells, bacteria require several features to survive inside host-cells, including: (i) avoid killing the host-cell and (ii) subvert intracellular host defences (Sendi and Proctor, 2009). Accordingly, SCVs can persist intracellularly through several adaptations.

(i) SCVs are well-adapted to intracellular survival by limiting their cytotoxicity. For this purpose, levels of functional RNAIII and *agr* expression in SCVs are very low compared to wild-type colonies, which results in a reduced expression of staphylococcal toxins such as α-toxin and TSST-1 (Proctor *et al.*, 2006). As a consequence, SCV-infected cells may remain viable (von Eiff *et al.*, 2001).

(ii) SCVs possess a thick cell wall and a reduced membrane potential, which confers some resistance against cationic antimicrobial peptides, an essential component of the host immune defence (Peschel, 2002). Moreover, due to the reduction activity of the TCA cycle and acetate catabolism in SCVs, carboxylate is shunted into the production of the polysaccharide intercellular adhesin (PIA), which confers resistance against the non-oxidative killing machinery of neutrophils (Al Laham *et al.*, 2007). In addition, SCVs resistance to antimicrobial peptides has specifically been demonstrated against

the thrombin-induced platelet microbicidal protein (Koo *et al.*, 1996) and lactoferrin B (Samuelsen *et al.*, 2005).

1.7.3. *S. aureus* phagosomal escape

Among its several mechanisms to subvert phagocytosis, phagosomal escape of *S. aureus* is an important strategy for intracellular survival (Lâm et al. 2010; Grosz et al. 2014) and such escape was detected after 2.5 hours of invasion in HEK293 cells (Grosz et al. 2014). The dependency of phagosomal escape on the Agr system has been demonstrated since *agr* knockout mutants are confined in the phagosome and not detected in the host cell cytosol (Blättner *et al.*, 2016; Münzenmayer *et al.*, 2016). PVL has also been described to play an important role for *S. aureus* phagosomal escape in keratinocytes (Chi *et al.*, 2014). In addition, the role of many other *S. aureus* toxins on phagosomal escape has been assessed with deletion mutants. Individual deletions of α-toxin, δ-toxin, β-toxin or PSMβ did not affect bacterial escape rates; however, PSMα was identified as a key factor in the phagosomal escape of *S. aureus* in both phagocytic and non-phagocytic cells (Grosz, Kolter, Paprotka, A. C. Winkler, *et al.*, 2014; Münzenmayer *et al.*, 2016). Nevertheless, PSMα expression alone is not sufficient to induce escape to the cytosol, and other phagosomal escape factors have been recently suggested (Blättner *et al.*, 2016), but further investigation is needed to fully understand their mechanism of action.

The specific subcellular location of *S. aureus* intracellular replication seems to be host cell-dependent. In non-phagocytic cells, *S. aureus* is able to proliferate in the host cell cytosol after phagosomal escape. However, autophagosomes have also been observed as a preferential niche for *S. aureus* replication (Schnaith *et al.*, 2007). In line with this

observation, the pharmacological control of autophagy can have a very significant impact on the intracellular survival of *S. aureus* in HeLa cells and fibroblasts (Schnaith *et al.*, 2007).

1.7.4. Host-cell death induced by *S. aureus*

Apoptosis is a programmed cell death mechanism, which is essential for cellular homeostasis in eukaryotic cells, and facilitates the eradication of damaged cells without promoting an inflammatory response. Other host cellular death mechanisms are necrosis and pyroptosis (Fraunholz and Sinha, 2012).

Some obligate intracellular pathogens, such as *Chlamydia* or *Ricketsia,* possess mechanisms to prevent host cell death in order to ensure their replication niche. By contrast, other intracellular pathogens, such as *Salmonella* or *Listeria,* induce host cell death mechanisms for different purposes, e.g. to overcome epithelial barriers or to kill immune cells. This pathogen-host cell death interplay may be quite complex, since most intracellular pathogens can initially promote host cell survival to guarantee intracellular replication, and then induce host cellular death at later points of the infection to facilitate bacterial cell dissemination (Horn *et al.*, 2017).

Specifically, *S. aureus* has shown to promote host cell death in a wide range of mammalian cells, including epithelial and endothelial cells, osteoblasts and professional phagocytes, such as macrophages and neutrophils. When *S. aureus*-induced cell death occurs, this has been mainly identified via apoptosis (Fraunholz and Sinha, 2012; Horn *et al.*, 2017), although necrosis induced by the α-toxin has also been reported in *S. aureus*-infected cells (Essmann *et al.*, 2003).

Regarding bacterial virulence factors involved in *S. aureus*-induced cell death, there is large variability depending on the host cell line as well as the bacterial strain tested. In fact, not all *S. aureus* strains have the ability to induce host cell death after cell infection (Krut *et al.*, 2003). Nevertheless, many bacterial factors have been commonly identified to be involved in the induction of host cell death. For example, the α-toxin and PVL are sufficient to directly induce leukocyte destruction (Otto, 2010; Berube and Wardenburg, 2013). Furthermore, PVL can also induce cell death in human and rabbit polymorphonuclear neutrophils (PMNs) by activation of caspase 9/3 signalling pathway (Genestier *et al.*, 2005; Chi *et al.*, 2014). Caspase 9 activates other effector caspases, which results in membrane blebbing and DNA fragmentation (Rudel, Kepp and Kozjak-Pavlovic, 2010). Additionally, caspase 2, which is induced by staphylococcal toxins such as α-toxin and aerolysin, is also involved in the initiation of caspase signalling that leads to cell death of non-professional phagocytes (Imre *et al.*, 2012).

Caspase-independent mechanisms have also been reported in *S. aureus*-induced host cell death. For example, c-JUN kinases (JNK) can be activated by inflammatory cytokines or cytotoxic agents to promote apoptosis by upregulation of pro-apoptotic proteins, and may also play an important role in *S. aureus*-induced cell death (Dhanasekaran and Reddy, 2008). Another cytotoxic factor involved in host cell death observed in *S. aureus* infected cells is PSMα (Haslinger-Löffler *et al.*, 2005; Rasigade *et al.*, 2013; Surewaard *et al.*, 2013).

On the other hand, the expression of *S. aureus* toxins may release cathepsin, which in turn activates the inflammasome resulting in host cell death via pyronecrosis (Muñoz-Planillo *et al.*, 2009). The staphylococcal leukocidin LukAB has also been correlated

to induce pyronecrosis, by activating the inflammasome components Caspase 1, NOD-like receptor protein 3 (NLRP3) and the adaptor protein ASC (Melehani *et al.*, 2015).

1.8. CLINICAL MANAGEMENT OF *S. AUREUS* INFECTIONS

Treatment of *S. aureus* infections is becoming a real challenge, especially considering the emergence of MRSA strains resistant to last resort of antibiotics, such as vancomycin (Howden *et al.*, 2010). Clinical management of MRSA infections varies depending on the type of infection as well as the bacterial strain. Overall, most MRSA infections usually require prolonged period of antibiotic therapy, hospitalization, and the removal of infected tissue or biomaterial in case of localized-infection or prosthetic joint infections, respectively (Tong et al. 2015).

The list of antibiotics currently available and approved to treat MRSA infections are vancomycin, daptomycin, linezolid and some other antimicrobials recently developed, including tedizolid, telavancin, oritavancin, dalbavancin, ceftaroline and ceftobiprole (Boswihi and Udo, 2018). Since these latter antibiotics are mostly employed to treat only SSTIs, the most commonly antibiotics used to treat MRSA infections are vancomycin, daptomycin and linezolid. However, these three last-resort antibiotics also present some limitations. For example, daptomycin cannot be used to treat pneumonia infections (Raja *et al.*, 2003), whereas linezolid is not suitable for bacteremia or infective endocarditis treatment (Watkins, Lemonovich and File Jr, 2012). In addition, strains with reduced susceptibility to daptomycin and linezolid, and Vancomycin-Intermediate or even Vancomycin-Resistant *S. aureus* (VISA & VRSA) have already been reported (Nannini, Murray and Arias, 2010). Furthermore, a recent

study showed that intracellular *S. aureus* is protected from antibiotics exposure, since intracellular killing is not achieved at clinically relevant concentrations of vancomycin, daptomycin or linezolid (Lehar *et al.*, 2015). This observation is also in line with previous studies, which reported that most antibiotics are not capable of intracellular *S. aureus* clearance *in vitro* (Barcia-Macay *et al.*, 2006) or *in vivo* (Sandberg *et al.*, 2009).

Additionally, it is becoming clear that the complex interaction between the pathogen and the immune system of the host is preventing the development of an efficient vaccine. For example, different staphylococcal virulence factors such as protein A possess immunosuppressive effects, which compromise the immunological memory of the host (Kobayashi and Deleo, 2013). Indeed, it has been recently demonstrated that vaccination against staphylococcal antigens located in the cell-surface may even aggravate the infection (Spaulding *et al.*, 2014). Another important issue regarding the development of a *S. aureus* vaccine is the use of experimental animals that are not natural hosts of this pathogen (Brown *et al.*, 2014). For instance, despite of some protection against *S. aureus* can be obtained in mice, the translation of this protective effect into humans has failed repeatedly (Kobayashi and Deleo, 2013).

Therefore, due to the alarming emergence of antibiotic resistance and the lack of a suitable vaccine against *S. aureus* infections, novel therapeutics capable of targeting intracellular bacteria, which have been shown to be protected from conventional antibiotics, are needed.

A recently explored strategy to tackle this problem is the design of antibodies conjugated with bactericidal antibiotics that are specifically released within the host cell (Lehar *et al.*, 2015). However, in this approach the antibiotics are only active due to the lower pH of the phagosome, suggesting that this antibody-antibiotic complex

may not be effective against cytosolic *S. aureus* or bacteria replicating in autophagosomes. In addition, the use of antibiotics targeting cell growth or division of the pathogen are a major selective pressure towards the emergency of resistant strains.

1.9. HOST-DIRECTED APPROACHES, A NEW PERSPECTIVE

A new strategy to develop novel therapies against intracellular pathogens is emerging in the form of host-directed therapies (Zumla *et al.*, 2016; Kaufmann *et al.*, 2018). Intracellular pathogens require the exploitation of host molecular factors and metabolic pathways to ensure their internalization, survival and proliferation within host cells. Therefore, host-directed approaches are focused on controlling the host determinants that intracellular bacteria exploit to support their intracellular life (Schwegmann and Brombacher, 2008). Future anti-infective therapies may consist in the combination of conventional antibiotics targeting bacterial survival outside of the host cell with host-directed therapies to efficiently eliminate intracellular pathogens.

Host-directed approaches require a deep understanding of the host-pathogen interactions underlying different intracellular processes. *S. aureus* has only been recently reconsidered as a facultative intracellular pathogen and therefore, our knowledge is still limited in this area. In contrast, the intracellular mechanisms as well as host metabolic alterations of other important pathogens, such as *Legionella pneumophila, Listeria monocytogenes, Mycobacterium tuberculosis, Salmonella enterica* or *Shigella flexnerii* have received much more attention. While some intracellular mechanisms are probably species-specific, we can achieve a better

understanding of the host-pathogen interplay in *S. aureus* infections by comparing it with other intracellular pathogens

In order to gain novel insights about host-pathogen interactions, individual or combined strategies that include -omics approaches (genomics, transcriptomics, proteomics and metabolomics) and computational biology could be particularly useful. Certainly, genome-wide screens employing RNA interference (RNAi), microarray analyses and combinatorial chemical libraries have already been employed to identify host genes required for bacterial entry and intracellular growth (Schwegmann and Brombacher, 2008).

RNAi approaches allow the development of loss-of-function phenotypic analyses to discover novel host-directed targets. Genome-wide RNA interference assays have been performed in *Drosophila* cells to identify host factors exploited and subverted by two intracellular pathogens: *Chlamydia caviae* and *L. monocytogenes*. In *C. caviae* infections, the depletion of human genes *Tomm40* or *Tomm22*, which are involved in protein transport to the mitochondria, resulted in a reduction of intracellular bacteria within mammalian cells (Derré *et al.*, 2007). Moreover, 116 host genes were identified to be important during *L. monocytogenes* infection and implicated in entry, bacterial escape and intracellular replication (Cheng *et al.*, 2005). Another example is the host-SKIP protein and its role in *Salmonella* infection discovered by an RNAi approach. *S.* Typhimurium replicates in a membrane-bound compartment, known as *Salmonella*-containing vacuole (SCV), and targets the host protein SKIP, which in turns down-regulates kinesin recruitment to control vacuole dynamics (Boucrot *et al.*, 2005).

Interestingly, a study that employed a genetic approach combining RNAi and automated microscopy revealed the importance of serine-threonine protein kinases (Akt1) in the intracellular survival of several intracellular pathogens. Akt1 kinase acts

as a key regulator of several guanosine triphosphatases (GTPases), which are involved in essential host pathways exploited by intracellular pathogens. For instance, *S.* Typhimurium is able to activate host Akt1 to control actin dynamics via p21(Cdkn1a)-activated kinase 4 (PAK4) and hence, facilitating its intracellular survival. *Salmonella* also controls phagosome-lysosome fusion through Akt1-Rab41 pathway (Kuijl *et al.*, 2007).

The use of metabolomics also represents an interesting approach to gain a better understanding of the metabolic scenario upon intracellular bacterial infection (Kint *et al.*, 2010; Jean Beltran *et al.*, 2017). It has been shown that several intracellular pathogens trigger host cell metabolism to support its intracellular survival (Eisenreich *et al.*, 2013). For example *L. pneumophila* interacts with the host mitochondria, inducing a Warburg-like effect in the host cell to favour its own replication (Escoll *et al.*, 2017). *S. flexneri* re-routes host central carbon metabolism through the glycolytic pathway to obtain an abundant nutrient-flux that allows its intracellular survival (Kentner *et al.*, 2014). Moreover, intracellular *M. tuberculosis* requires host cholesterol import to persist inside both macrophages and mice´s lungs (Pandey and Sassetti, 2008). Similarly, a recent study investigated the host metabolic response in a human airway epithelial cells after *S. aureus* infection. They observed a reduction in nutrient uptake as well as nucleotide biosynthesis in *S. aureus*-infected cells (Gierok *et al.*, 2016), although the underlying mechanisms are still unclear.

Once specific host factors or metabolic pathways required by the intracellular pathogen have been described, blocking those pathways – e.g. by using drug inhibitors – may be considered as a novel strategy to control bacterial infections. For example, Akt1 and protein kinase A (PKA) inhibitors have shown to reduce intracellular load

of *M. tuberculosis* and *S.* Typhimurium in infected human macrophages (Kuijl *et al.*, 2007).

In particular, several studies have already shown that existing and approved drugs, which are used for other clinical purposes unrelated to antimicrobial activity, can influence the outcome of bacterial infections. The main advantage of finding "repurposed drugs" is that they are already clinically approved, shortening the time to reach the clinic (Schwegmann and Brombacher, 2008; Czyż *et al.*, 2014). An example of repurposed drug is the histone deacetylase inhibitor Sulforaphane, which induces the expression of leukocyte protease inhibitor and β-defensin 2 and therefore, increases the activity of antibiotics against the multidrug-resistant strain *Neisseria gonorrhoeae* (Yedery and Jerse, 2015).

Moreover, host-directed therapies can also aim to enhance host cellular responses against intracellular pathogens, to activate innate and adaptive host immune responses or to modulate disproportionate inflammation. Hence, host-directed therapies also include immunomodulatory agents – including monoclonal antibodies or nutritional products – as well as cellular therapy (Zumla *et al.*, 2016). For example, monoclonal antibodies anti-TNFα or anti-interleukins, which reduce tissue-destructive inflammation by cytokine neutralisation, have shown a bactericidal effect against the intracellular pathogens *M. tuberculosis* and *Helicobacter pylori* (Wallis, van Vuuren and Potgieter, 2009; Wroblewski, Peek and Wilson, 2010; Rossi *et al.*, 2015). Further, the nutritional product vitamin D3 is also effective against these two pathogens and its mechanism of action has been described as an activation and enhancement of host antimicrobial defences (Guo *et al.*, 2013; Rahman *et al.*, 2015).

Some of the host-directed drugs that have shown positive effects against intracellular bacterial pathogens are listed in table 1.2. These examples provide proof-of-principle

about the potential of host-directed approaches to improve the outcome of bacterial infections caused by intracellular pathogens. Such therapies have shown the ability to achieve intracellular killing and they also possess the benefit of preventing the development of antimicrobial resistance. In particular, many host-directed drugs have been identified against *M. tuberculosis* and they are already under clinical trials (Reviewed in Zumla et al. 2016; Kaufmann et al. 2018).

Class	Drug	Mechanism of action	Pathogen	Reference
Monoclonal antibody	Adalimumab	anti-TNFα	*Mycobacterium tuberculosis*	(Wallis et al. 2009)
	Anti-interleukin 1β	Cytokine neutralisation	*Helicobacter pylori*	(Wroblewski et al. 2010)
	Antipertussis toxins antibody	Enhancement of immunoglobulins	*Bordetella pertussis*	(Scanlon et al. 2015)
	anti-TNFα	Cytokine neutralisation	*Helicobacter pylori*	(Wroblewski et al. 2010)
	Bevacizumab	anti-VEGF	*Mycobacterium tuberculosis*	(Datta et al. 2015; Oehlers et al. 2015)
	Nivolumab	anti-PD-1	*Mycobacterium tuberculosis*	(Singh et al. 2013; Jurado et al. 2008)
	Siltuximab	anti-interleukin 6	*Mycobacterium tuberculosis*	(Ravimohan et al. 2015)
Recombinant protein	β-defensin 2	Host-antimicrobial peptide	*Neisseria gonorrhoeae*	(Leduc et al. 2015)
Repurposed drug	Aspirin	NSAID, TNFα levels reduction	*Mycobacterium tuberculosis*	(Tobin et al. 2013)
			Staphylococcus aureus	(Bleiss et al. 2004)
	Chlorpromazine	Calmodulin antagonist	*Neorickettsia risticii*	(Rikihisa et al. 1995)
			Salmonella enterica serovar Typhimurium	(Amaral et al. 2000)
	ETB067	Akt1 inhibitor	*Mycobacterium tuberculosis*	(Kuijl et al. 2007)
	Fingolimod	Activation of sphingosine-1-phosphate pathway	*Bordetella pertussis*	(Skerry et al. 2015)
	Glibendamide	Cyclooxygenase inhibition	*Streptococcus pneumoniae*	(Koh et al. 2013)
	H-89	PKA inhibitor	*Salmonella typhimurium*	(Kuijl et al. 2007)
			Coxiella burnetii	(Czyż et al. 2014)

Class	Drug	Mechanism of action	Pathogen	Reference
Repurposed drug	Ibuprofen	NSAID, Cyclooxygenase inhibition	*Streptococcus pneumoniae*	(Bernard et al. 1997)
			Mycobacterium tuberculosis	(Vilaplana et al. 2013; Ivanyi et al. 2013)
	Imatinib mesylate	BCR-ABL tyrosine kinase inhibitor	*Mycobacterium tuberculosis*	(Napier et al. 2011)
			Anaplasma phagocytophilum	(Lin et al. 2007)
	Indometacin	Cyclooxygenase inhibition	*Streptococcus pneumoniae*	(Hanly et al. 1987)
	Metformin	Mitochondrial respiratory chain blocker	*Mycobacterium tuberculosis*	(Singhal et al. 2014)
	Niraparib	PARP inhibitor	*Mycobacterium tuberculosis*	(Pirinen et al. 2014)
	Phenylbutyrate	Histone deacetylase inhibitor	*Mycobacterium tuberculosis*	(Coussens et al. 2015)
			Listeria monocytogenes	(Lieberman & Higgins 2008)
	Pimozide	Calcium channel inhibitor	*Bacillus subtilis*	(Lieberman & Higgins 2008)
			Salmonella enterica serovar Typhimurium	(Lieberman & Higgins 2008)
			Escherichia coli	(Lieberman & Higgins 2008)
	Prednisone	Glucocorticoid receptor antagonist	*Streptococcus pneumoniae*	(Blum et al. 2015)
			Mycobacterium tuberculosis	(Critchley et al. 2013)
	Raloxifene	Oestrogen receptor modulator	*Pseudomonas aeruginosa*	(Ho Sui et al. 2012)
	Statins	Cyclooxygenase inhibition	*Streptococcus pneumoniae*	(Mortensen et al. 2008, Chalmers et al. 2008)
			Mycobacterium tuberculosis	(Parihar et al. 2014)
	Sulforaphane	Histone deacetylase inhibitor	*Neisseria gonorrhoeae*	(Yedery et al. 2015)
	Thapsigargin	Calcium ATPase inhibitor	*Coxiella burnetii*	(Czyż et al. 2014)

Class	Drug	Mechanism of action	Pathogen	Reference
Repurposed drug	Thioridazine	unknown	*Listeria monocytogenes*	(Lieberman & Higgins 2010)
			Staphylococcus aureus	(Bleiss et al. 2004)
			Mycobacterium tuberculosis	(Amaral et al. 2017)
	Verapamil	Calcium channel inhibitor	*Mycobacterium tuberculosis*	(Martins 2008; Gupta et al. 2015)
			Chlamydia pneumoniae	(Salin et al. 2011)
	Vorinostat	Histone deacetylase inhibitor	*Mycobacterium tuberculosis*	(Shan et al. 2012)
	Zileuton	Leukotriene synthesis inhibitor	*Mycobacterium tuberculosis*	(Mayer-Barber et al. 2014)
Vitamin	Vitamin D3	Activation of antimicrobial defences	*Helicobacter pylori*	(Guo et al. 2014)

Table 1.2. List of host-directed drugs able to halt the intracellular infection caused by different pathogens

1.10. JUSTIFICATION AND AIMS

S. aureus is an opportunistic and versatile human pathogen responsible for a wide range of diseases worldwide (Lowy, 1998; Nelson *et al.*, 2015). Besides its pathogenic capacity, it is also a commensal microorganism and approximately 30% of the global population is asymptomatically and permanently colonized by MSSA or MRSA strains (Wertheim *et al.*, 2005). Importantly, persistent nasal colonization has been associated with recurrent staphylococcal infections, increased mortality rates and healthcare costs (Sollid *et al.*, 2014; Sakr *et al.*, 2018).

The versatility of *S. aureus* is characterized by its large plethora of virulence factors and, consequently, its ability to invade a wide range of host cells and tissues (Gordon and Lowy, 2008). For initial invasion and colonization, *S. aureus* possesses various surface-associated proteins that are capable of binding different host-surfaces and receptors (Foster and Höök, 1998). It can also secrete a wide range of proteins, toxins and enzymes, that support bacterial replication and later dissemination (Otto, 2014).

Furthermore, the ability of *S. aureus* to acquire antibiotic resistance has been extensively reported (Lowy, 2003). The emergence of MRSA strains dates back from 1960s and since then, *S. aureus* has evolved resistance to multiple antibiotics and, in particular, HA-MRSA strains are resistant to most available antibiotics (Naimi *et al.*, 2003). The last-resort antibiotics commonly used to treat MRSA infections are daptomycin, linezolid and vancomycin. However, reduced susceptibility to daptomycin and linezolid, as well as the emergence of vancomycin-intermediate and vancomycin-resistant *S. aureus* strains (VISA & VRSA) have already been shown (Nannini, Murray and Arias, 2010).

In addition to its virulence determinants and the increase of multidrug-resistant strains, *S. aureus* is also able to survive and proliferate within mammalian cells, to subvert phagocytosis and to induce host-cell death (Fraunholz and Sinha, 2012; Horn *et al.*, 2017). Moreover, *S. aureus* has been shown to produce biofilms, which enhance its intracellular persistence, thereby contributing to chronicity of infections and later dissemination from initial site of infection to secondary loci (Kong, Vuong and Otto, 2006). The intracellular state of *S. aureus* may protect this pathogen from decolonization procedures as well as antibiotic pressure (Horn *et al.*, 2017). Accordingly, it has recently been shown that the last resort antibiotics daptomycin, vancomycin and linezolid are unable to achieve intracellular killing (Lehar *et al.*, 2015).

Considering the increase of multidrug-resistant *S. aureus* strains as well as its intracellular protection against the currently available antibiotics, there is an urgent need to investigate novel therapeutic approaches. Traditional therapies have been focused on targeting bacterial cell growth and cell division. The main advantage of pathogen-directed therapies is their limited cytotoxicity, but the use of these treatments quickly leads to the emergence of multidrug-resistant strains (Schwegmann and Brombacher, 2008). Host-directed approaches offer the opportunity to overcome the problem of antibiotic resistance and to achieve intracellular killing. They target host-molecular pathways that intracellular bacteria exploit to support survival and replication within mammalian cells (Zumla *et al.*, 2016; Kaufmann *et al.*, 2018).

Thus, this study aimed to characterize the host molecular factors and metabolic pathways hijacked by *S. aureus* during intracellular infection in order to discover novel therapies against this versatile pathogen. At the same time, this thesis

provides novel insights into *S. aureus*-host interactions, contributing to a better understanding of intracellular staphylococcal infections.

Specifically, I employed three different approaches to identify the host cellular pathways exploited by intracellular *S. aureus* and these approaches resulted in different result chapter as follows:

(i) First, I characterized the host-metabolic changes upon intracellular staphylococcal infection by using a metabolomic approach.

(ii) Second, I screened a panel of host-directed drugs in order to find "repurposed drugs" that may have a potential effect against intracellular *S. aureus*.

(iii) Lastly, I pursued the identification of novel host-genes that are involved in intracellular invasion and/or proliferation. By using a genome-wide shRNA screening, we assessed the effect of silencing human genes on *S. aureus* infection.

2. Materials and Methods

2.1. MICROBIOLOGY

2.1.1. Bacterial strains and culture conditions

All bacterial strains used in this study are listed in table 2.1. *S. aureus* strains were cultured in Nutrient Broth (NB) medium (Sigma-Aldrich) and *E. coli* strains were grown in Luria Broth (LB) medium (Sigma-Aldrich). For growth in liquid culture, bacterial strains were incubated at 37°C, with vigorous shaking (300 rpm) in an Incu-shake MIDI 4020 incubator (SciQuip). For growth on solid medium, Nutrient agar (Sigma-Aldrich) and LB agar (Sigma-Aldrich) plates were prepared following manufacturer's instructions and employed for *S. aureus* and *E. coli* strains' growth, respectively. Agar plates were incubated in a 37°C incubator (LMS). When necessary, medium was supplemented with ampicillin (100 μg/ml) for *E. coli* growth and chloramphenicol (7.5 μg/ml) for *S. aureus* growth, as indicated in table 2.1.

2.1.2. Preparation of bacterial stock

A single colony from a fresh agar plate was used to inoculate 10 ml of NB or LB medium and liquid culture was incubated overnight with shaking (37°C; 300 rpm). Next day, the overnight culture was aliquoted in 1 ml cryovials (Thermo Fisher Scientific) containing 20% glycerol and aliquots were stored at -80°C.

2.1.3. Bacterial pre-inoculum preparation for intracellular assays

S. aureus USA300 and NCTC 13626 strains were grown in 10 ml of NB at 37°C with shaking (300 rpm) overnight and 0.5 ml of this overnight culture was used to inoculate a flask containing 50 ml of NB (dilution 1:100). Bacterial culture was grown until an optical density (OD_{600nm}) of 1 was reached, upon which the culture was centrifuged (4,000 rpm, 15 min, 4ºC). Pellets were then washed twice with PBS, resuspended in 1.5 ml of PBS supplemented with 20% glycerol and aliquots of 100µl were stored at -80ºC until further use. The concentration of each preinocula was calculated the next day by serial dilution plating and Colony Forming Units (CFU) counting.

2.1.4. *In-vitro* growth assays

Bacterial strains were grown overnight in NB liquid medium at 37°C, with shaking (300 rpm). The overnight culture was diluted (1:100) in fresh NB medium and aliquots of 90 µl were dispensed per well in a 96-wells plate (Sarstedt). When different conditions were assessed, 10 µl of either drug or antibiotic was added to

each well. To evaluate bacterial growth, optical density (OD_{600nm}) was measured at time points 0, 2, 4, 6, 8 and 24 hours by using EL800 Microplate reader (Bio-Tek).

Table 2.1. List of bacterial strains used in this study

Strains	Description	Source
S. aureus		
MRSA USA300 LAC	LAC strain of the CA-MRSA USA300 lineage	Dr. Andrew Edwards (Edwards, 2012)
MRSA NCTC 13626	HA-MRSA strain originally isolated at St Thomas' Hospital London	NCTC
Lac*pcL55-ptet-gfpmut2	*S. aureus* USA300 strain expressing constitutively the green fluorescent protein (GFP). Chloramphenicol resistant (7.5 µg/ml) in LB agar plates.	Dr. Angelika Grundling (Reichmann *et al.*, 2014)
E. coli		
DH5-α-Select Gold	Host strain for molecular cloning. Resistant to ampicillin (100 µg/ml).	Bioline
DH5-α _pCR2.1-TOPO-mChe-TRAM2	DH5-α strain carrying the plasmid pCR2.1-TOPO-mChe-TRAM2. Resistant to ampicillin (100 µg/ml).	This study
DH5-α _pCR2.1-TOPO- mChe-SaCWT	DH5-α strain carrying the plasmid pCR2.1-TOPO-mChe-SaCWT-IRES. Resistant to ampicillin (100 µg/ml).	This study
DH5-α _P12-MMP-TRAM2-mChe-IRES-Hyg	DH5-α strain carrying the plasmid P12-MMP-mChe-TRAM2-IRES-Hyg. Resistant to ampicillin (100 µg/ml).	This study
DH5-α _P12-MMP- mChe-LC3A-IRES-Hyg	DH5-α strain carrying the plasmid P12-MMP-mChe-LC3A-IRES-Hyg. Resistant to ampicillin (100 µg/ml).	This study
DH5-α _P12-MMP- mChe-SaCWT-IRES-Hyg	DH5-α strain carrying the plasmid P12-MMP-mChe-SaCWT-IRES-Hyg. Resistant to ampicillin (100 µg/ml).	This study

2.2. CELL CULTURE

2.2.1. Cell lines and culture conditions

All cell lines employed in this study are listed in table 2.2 and every cell line was incubated at 37°C and 5% of CO_2 in a Heraeus® BB15 incubator (Thermo Scientific).

HeLa cells (ECACC) and HEK293T cells were grown in Dulbecco´s Modified Eagle´s medium (DMEM, Gibco) containing pyruvate, glucose and glutamine and supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gibco) and 5% of penicillin and streptomycin solution (Gibco), unless otherwise specified. When stated, HeLa cells carrying the P12-MMP vector were cultured in DMEM medium supplemented with hygromycin (200 μg/ml).

BALB/c Mouse Bone Marrow Macrophages (Calltag Medsystems) were cultured on Complete Macrophage Medium supplemented with granulocyte-macrophage colony-stimulating factor, penicillin, streptomycin and FBS (Calltag Medsystems).

Human Umbilical Vein Endothelial Cells (HUVEC, Sigma-Aldrich) were grown in Endothelial Cell Growth Medium (Sigma-Aldrich), supplemented with 5% of penicillin and streptomycin solution (Gibco), unless otherwise stated.

For the ^{13}C isotope labelling experiment for Gas-Chromatography Mass-Spectrometry (GC-MS), Minimum Essential Media (MEM; Gibco) – containing 10% of heat-inactivated FBS (Gibco) – was supplemented with either labelled or unlabelled glucose (5.6 mM) or glutamine (2 mM) as described below:

I. MEM supplemented with both unlabelled glucose (Gibco) and glutamine (Gibco).

II. MEM supplemented with $^{13}C_6$-labelled glucose (Cortecnet) and unlabelled glutamine.

III. MEM supplemented with $^{13}C_5$&$^{15}N_2$-labelled glutamine (Cortecnet) and unlabelled glucose.

Table 2.2. List of cell lines used in this study

Cell lines	Description	Source
HeLa	Human cervix epithelial carcinoma cells	ECACC
BALB/c Mouse Bone Marrow Macrophages	Primary mouse bone marrow cells	Caltag Medsystems
HEK-293T	Human embryonic kidney SV40 transformed cells. Used for the production of retrovirus to transduce HeLa cells	Dr. Yolanda Calle
HUVEC	Human Umbilical Vein Endothelial cells.	Sigma
HEK-293T EPHA2 KO	EPHA2 knockout cell lines derived from HEK-293T cells	Prof. Richard Longnecker (Chen *et al.*, 2018)
HeLa_ P12-MMP-TRAM2-mChe-IRES-Hyg	HeLa cells carrying the plasmid P12-MMP-mChe-TRAM2- IRES-Hyg on their chromosome. Resistant to hygromycin (200 µg/ml)	This study
HeLa_ P12-MMP-mChe-LC3A-IRES-Hyg	HeLa cells carrying the plasmid P12-MMP-mChe-LC3A- IRES-Hyg on their chromosome. Resistant to hygromycin (200 µg/ml)	This study
HeLa_ P12-MMP- mChe-SaCWT-IRES-Hyg	HeLa cells carrying the plasmid P12-MMP-mChe-SaCWT- IRES-Hyg on their chromosome. Resistant to hygromycin (200 µg/ml)	This study

2.2.2. Transduction of mammalian cells

For the shRNA screening, HeLa cells were transduced with the Mission® LentiPlex Human shRNA Library (Sigma-Aldrich). This library is comprised by 10 sub-pools of lentiviral particles carrying around 75,000 shRNA constructs that target approximately 16,000 human genes. HeLa cells were seeded in ten different 10 cm plates (Sarstedt) in complete DMEM medium at a cell density of $2x10^6$ cells per dish and placed them into the incubator overnight (37ºC, 5% CO_2). The following day, the ten lentiviral sub-pools were thawed on ice and added to HeLa cells at a Multiplicity of Infection (MOI) of 1, in the presence of hexadimethrine bromide (8 μg/ml; Sigma-Aldrich). After an overnight incubation, the virus-containing media were replaced with fresh DMEM, without hexadimethrine bromide. The next day, HeLa cells were exposed to puromycin selection (1 μg/ml; Sigma-Aldrich) and medium was replaced with fresh puromycin-containing media every two days until only positive transduced-cells remained.

To produce individual knockdowns, HeLa cells were transduced with 29 individual shRNA lentiviral constructs (Table 2.3, Sigma-Aldrich). HeLa cells were seeded in 24 wells-plate at a cell density of $8x10^4$ cells per well and incubated overnight (37ºC, 5% CO_2). Transduction of mammalian cells was performed as described above, employing a MOI of 1.

Table 2.3.List of lentiviral particles employed to produce individual knockdowns in HeLa cells

Clone ID	Symbol	Gene description
TRCN0000127872	ATG10	ATG10 autophagy-related 10 homolog
TRCN0000122869	AVEN	Apoptosis; caspase activation inhibitor
TRCN0000199388	BCKDK	Branched chain ketoacid dehydrogenase kinase
TRCN0000130856	CCDC82	Coiled-coil domain containing 82
TRCN0000057589	CD5L	CD5 molecule-like
TRCN0000056919	CD83	CD83 molecule
TRCN0000056647	CDKAL1	CDK5 regulatory subunit associated protein 1
TRCN0000055994	CLTB	Clathrin; light polypeptide (Lcb)
TRCN0000058248	CSF2RA	Colony stimulating factor 2 receptor alpha; low affinity
TRCN0000142964	FAM63B	Family with sequence similarity 63, member B
TRCN0000129900	IFIT1B	Interferon-induced protein with tetratricopeptides repeats 1-like
TRCN0000044726	KCNK1	Potassium channel; subfamily K; member 1
TRCN0000055585	MEGF9	Multiple epidermal growth factor-like; domain 9
TRCN0000053959	MYL2	Myosin; Light polypeptide 2; regulatory; cardiac; slow
TRCN0000053505	MYL9	Myosin; light polyeptide 9; regulatory
TRCN0000054308	NELL2	NEL-like 2
TRCN0000138344	NSUN7	NOL1/NOP2/Sun domain family; member 7
TRCN0000144974	ORC1L	Origin recognition complex; subunit 1-like
TRCN0000053075	POLA2	Polymerase (DNA directed); alpha 2
TRCN0000056506	RAB11FIP4	RAB11 family interacting protein 4 (Class II)
TRCN0000047220	RAB3GAP2	RAB3 GTPase activating protein subunit 2 (non-catalytic)
TRCN0000141304	RAB41	RAB41; member RAS; oncogene family
TRCN0000044061	SLC36A3	Solute carrier family 36 (proton/amino acid symporter), member 3
TRCN0000044314	SLC43A1	Solute carrier family 43; member 1
TRCN0000057018	TLR2	Toll-like receptor 2
TRCN0000059251	TNFRSF10C	Tumor necrosis factor receptor superfamily; member 10C
TRCN0000141247	TRAM2	Translocation associated membrane protein 2
TRCN0000011171	UBXN10	UBX domain containing 3
TRCN0000137602	ZNF618	Zinc finger protein 618

2.2.3. Intracellular infection assays

Different intracellular infections were performed for different purposes in this study and therefore, the type of plate or pre-inoculum's strain that was employed as well as the number of cells that were seeded varies among experiments (such variables are summarised in table 2.4).

For mammalian DNA extraction and further sequencing, HeLa cells stably expressing the shRNA library, were seeded in 10 cm cell culture dish (Sarstedt) in DMEM without antibiotics, at a cell density of $2x10^6$ cells per dish. For metabolomics approaches, HeLa cells were seeded in 6-well plates (Sarstedt) in DMEM without antibiotics and at a cell density of $5x10^5$ cells per well. For host-cell viability and immunofluorescence assays, HeLa cells and individual knockdowns produced in HeLa cells were seeded in 24-well plates (Sarstedt) in DMEM without antibiotics and at cell density of $7.5x10^4$ cells per well. For the drug-screening and host cell viability experiments, HeLa cells and HUVEC were seeded in 96-wells plate at cell density of $1.8x10^4$ cells per well. In all cases, cells were allowed to settle in the incubator (37°C, 5% CO_2) for an overnight.

The next day, an aliquot of bacterial pre-inoculum was thawed, diluted in 900 µl of Dulbecco's Phosphate-buffered saline (PBS; Sigma-Aldrich) and centrifuged at 4,000 rpm for 5 minutes. Pellet was washed twice with PBS prior to addition of DMEM without antibiotics at a bacterial density that corresponds to a MOI of 100. Then, bacterial suspension was added to each well, plates were immediately centrifuged at 1,500 rpm for 5 minutes and incubated at 37ºC in 5% CO_2 for 45 minutes to allow bacterial internalization. When heat-killed condition was included, bacterial pre-inoculum was incubated for 10 min at 95ºC prior to centrifugation.

The medium was then replaced by DMEM supplemented with either 100 µg/ml gentamycin or 5 µg/ml vancomycin depending on the strain employed (Fig. 2.1) and cells were incubated for 1 hour to kill extracellular bacteria. After one hour of incubation, the medium was replaced again by DMEM supplemented with 10 µg/ml gentamycin or 5 µg/ml vancomycin and cells were placed back in the incubator (37ºC, 5% CO_2) for four additional hours, until time point 6 was reached.

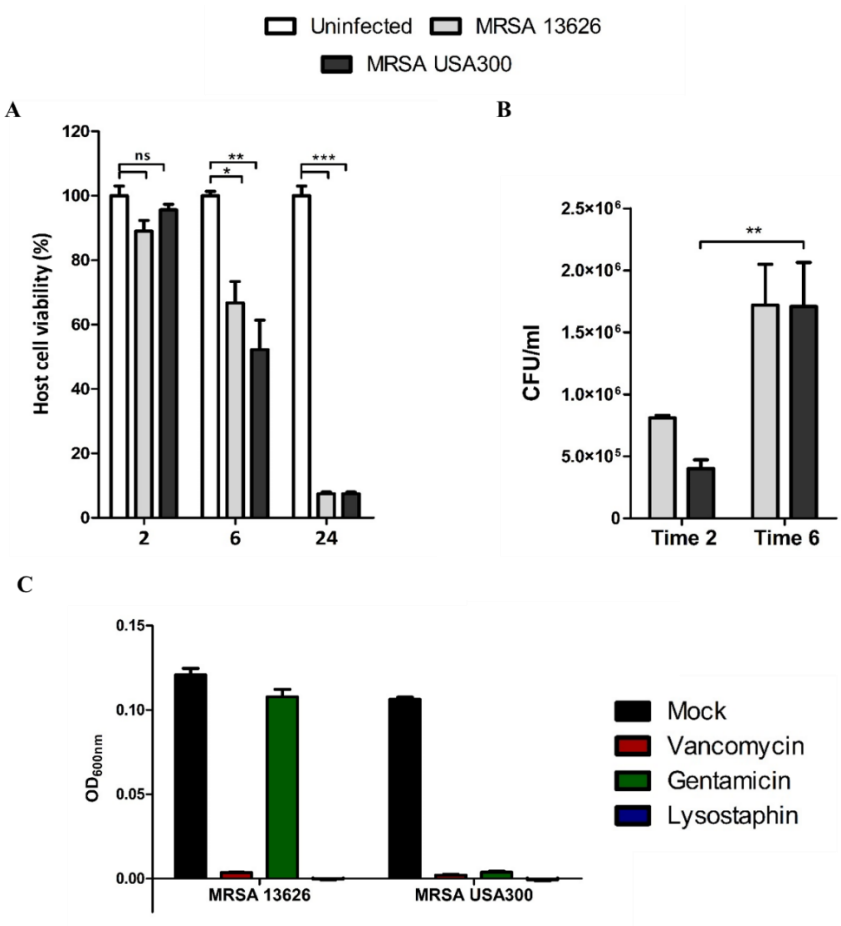

Figure 2.1. Analysis of host cell viability and intracellular bacterial survival during *S. aureus* USA300 and NCTC 13626 infection assays, and their resistance to gentamycin, vancomycin and lysostaphin. (A) Quantification of host cell viability at different time points of infection, measured by flow cytometry using double annexin V-FITC and PI staining. (B) Intracellular survival of USA300 and NCTC13626 at different time points; HeLa cells were infected with *S. aureus* and lysed in 0.1% Triton X-100 for CFU evaluation. (C) Bacterial growth levels at 6 hours in NB medium supplemented with gentamycin (100 µg/ml), vancomycin (5 µg/ml) and lysostaphin (5 µg/ml). Graphs display means ± standard error of three independent experiments. Statistical significance was analysed by one-way ANOVA and post hoc Tukey's multiple comparison tests. p-value ≤0.05 (*); ≤0.01 (**); ≤0.001 (***).

2.2.4. Host cell viability assay

Cell infection was performed as described above and samples for host cell viability quantification were collected after six hours of MRSA infection. In order to recover both necrotic and apoptotic cells, the medium of each well was aspirated and transferred into clean Eppendorf's tubes (Fisher Scientific). Cells were trypsinized for 5 minutes, diluted in DMEM without antibiotics and centrifuged at 1,500 rpm for 10 minutes. Afterwards, cells were double stained with 2.5 µl of annexin V-FITC and 5µl of propidium iodide (PI) (Becton Dickinson, BD) diluted in 50 µl of DMEM per sample and incubated for 15 minutes at room temperature. Stained cells were centrifuged at 1,500 rpm for 10 minutes, supernatant aspirated and cell pellets were fixed using 150 µl of BD Cytofix buffer (Becton Dickinson, BD) for 15 minutes at 4°C. Finally, samples were diluted with 350 µl of DMEM without antibiotics and host cell viability was measured by flow cytometry (BD Accuri^{TM} C6 Plus).

For the high-throughput drug-screening, a modified protocol was applied. After infection was performed in 96-wells plate and time 6 was reached, plate was centrifuged at 1,200 rpm for 10 minutes and supernatant was discarded, retaining only alive cells that are attached to the surface of the well. Afterwards, cells were fixed using 20 µl of BD Cytofix buffer (Becton Dickinson, BD) for 15 minutes at 4°C and samples were diluted with 100 µl of DMEM without antibiotics. Host cell viability was estimated in the flow cytometer by counting the number of events within an alive-gating in 1 min per sample.

2.2.5. Intracellular bacterial estimation

When USA300 wild type strain was employed for intracellular infection assays, cells were lysed using 0.1% Triton X-100 diluted in PBS (5 min, RT) and serial dilutions were plated in NB agar plates for CFU counting. For the high-throughput drug-screening, where USA300-GFP bacterial strain was employed, intracellular bacterial survival was calculated by measuring the percentage of GFP in the flow cytometer.

Table 2.4. Experimental specifications for the different intracellular infection assays of this study

Experiment	Plate	Numbers of cells seeded	Bacterial strain	Bacterial pre-inocula	Antibiotic
shRNA screening	10 cm TC dish	$2x10^6$ cells/well	MRSA NCTC 13626	$2.1x10^9$ CFU/ml	Vancomycin
Metabolomics assays	6-wells plate	$5x10^5$ cells/well	MRSA NCTC 13626	$2.1x10^9$ CFU/ml	Vancomycin
	6-wells plate	$5x10^5$ cells/well	MRSA USA300	$1.8x10^9$ CFU/ml	Gentamicin
Host cell viability assays	24-wells plate	$7.5x10^4$ cells/well	MRSA NCTC 13626	$2.1x10^9$ CFU/ml	Vancomycin
	24-wells plate	$7.5x10^4$ cells/well	MRSA USA300	$1.8x10^9$ CFU/ml	Gentamicin
Immunofluorescence assays	24-wells plate	$7x10^4$ cells/well	MRSA USA300-GFP	$8.7x10^8$ CFU/ml	Gentamicin
Drugs screening	96-wells plate	$1.8x10^4$cells/well	MRSA USA300-GFP	$8.7x10^8$ CFU/ml	Gentamicin

2.3. MOLECULAR BIOLOGY

2.3.1. Polymerase chain reaction (PCR)

Polymerase chain reactions (PCRs) were carried out in T100™ Thermal Cycler (Bio-Rad). The oligonucleotides used in this study (table 2.5) were purchased from Thermo Fisher Scientific. Oligonucleotides were reconstituted in molecular grade pure water (Sigma-Aldrich) to a final concentration of 100 mM. Before establishing PCR cycling conditions, oligonucleotide's annealing temperatures were calculated using the OligoCalc webpage (http://biotools.nubic.northwestern.edu/OligoCalc.html).

For molecular cloning purposes, Phusion™ High Fidelity DNA polymerase (Thermo Fisher Scientific) was employed. A 50 µl master mix volume was prepared containing: 10 µl of 5X Phusion HF Buffer, 1 µl of dNTPs (10 mM), 2.5 µl of each oligonucleotides (10 mM), 2.5 µl of DMSO unless otherwise stated, 2 µl of template DNA and 0.5 µl of Phusion DNA Polymerase. Master Mix reaction was mixed well by pipetting and spinned briefly before placing in the Thermal Cycler (BioRad). PCR cycling conditions were as follows: 30 seconds of initial denaturation at 98°C, 25 cycles of amplification which involves 10 seconds of denaturation at 98°C, 30 seconds of oligonucleotide annealing at the corresponding melting temperature and 30 seconds of elongation per kilo base (kb) at 72°C and another 10 extra minutes of final elongation at 72°C at the end of the cycle.

For routine PCR checking, 5Prime HotMaster mix polymerase (QuantaBio) was used. A 10 µl master mix volume was prepared containing: 0.5 µl of each oligonucleotides (10 mM), 3 µl of template DNA and 4 µl of 5Prime HotMaster

mix polymerase. PCR cycling conditions were the same as stated above but 1 minute of elongation per kb was applied.

2.3.2. Agarose gel electrophoresis

PCR reactions were verified by visualization of DNA bands in 1% agarose electrophoresis gels. In order to make 1% agarose gels, one Topvision agarose tablet (Thermo Scientific) was diluted per 50 ml of Tris-Acetate-EDTA(TAE) buffer (40 mM Tris base, pH 7.6; 20mM acetic acid; 1mM EDTA) in a 250 ml Erlenmeyer flask. Agarose was melted in a microwave oven for 1-2 minutes at maximum power, until agarose was completely dissolved. Agarose solution was allowed to cool down for a few minutes at room temperature, SYBR safe DNA gel stain (Thermo Fisher Scientific) was diluted 1: 10,000 and agarose solution was poured in the gel tray. After 20-25 minutes of incubation at room temperature, the comb and tape from the edges of the solidified gel tray were carefully removed and gel tray was placed on the Sub-Cell tank (Bio-Rad), filled with TAE buffer.

To load the samples, each 5 µl of DNA was mixed with 1 µl of Orange Loading dye (Thermo Fisher Scientific) and carefully loaded to each well. 5-10 µl of a 1 kb DNA molecular weight ladder (Thermo Fisher Scientific) was added to the first wells. Electrophoresis gels were run at a constant voltage of 90V for 30 minutes – 1 hour using a PowerPac Basic Power Supply (Bio-Rad).

Table 2.5. List of oligonucleotides used in this study for molecular cloning. Restriction enzymes are underlined in the sequence

Experiment	Primer name	5' → 3' sequence	Purpose
Molecular construction of P12-MMP-TRAM2-mChe-IRES-Hyg	TRAM2-HindIII FW	tagcta aagctt gccacc ATGGCTTTCCGCAGGAGG	To amplify TRAM2 gene (forward), including a restriction enzyme site (HindIII)
	TRAM2-mChe RV	GCCCTTGCTCACCAT GGGAGACTTGAGTTT	To amplify TRAM2 gene (reverse)
	TRAM2-mChe FW	AAACTCAAGTCTCCC ATGGTGAGCAAGGGC	To amplify mCherry (forward)
	mChe-NotI RV	tagcta gcggccgc ttta CTTGTACAGCTCGTCCAT	To amplify mCherry (reverse), including a restriction enzyme site (NotI)
Molecular construction of P12-MMP-mChe-SaCWT-IRES-Hyg	mChe-HindIII FW	tagcta aagctt gccacc ATGGTGAGCAAGGGCGAG	To amplify mCherry (forward), including a restriction enzyme site (HindIII)
	mChe-SaCWT RV	TGATGGGCTCGAGCCCTTGTACAGCTCGTC	To amplify mCherry (reverse)
	mChe-SaCWT FW	GACGAGCTGTACAAGGGCTCGAGCCCATCA	To amplify SaCWT (forward)
	SaCWT-NotI RV	tagcta gcggccgc ttta CTTTATAGTTCCCCAAAG	To amplify SaCWT (reverse), including a restriction enzyme site (NotI)

2.3.3. DNA extraction

2.3.3.1. Plasmid extraction

All plasmids used in this study are stated in table 2.6. For plasmid extraction, one colony of the *E. coli* strain was taken to inoculate 10 ml of LB liquid medium, supplemented with the appropriate antibiotic, depending on the plasmid, and incubated overnight with shaking (37°C, 300 rpm). Next morning, plasmid extractions were performed using Monarch® Plasmid Miniprep kit (New England Biolabs, NEB) and manufacturer's guidelines were followed.

2.3.3.2. Bacterial DNA extraction

When PCR reaction produced only one clear band, the GeneJET DNA clean-up kit (Thermo Fisher Scientific) was used to extract DNA, following manufacturer's instructions. When several bands were visualized in the electrophoresis gel, the band of interest was cut out from the agarose gel and DNA was extracted from the agarose by using GeneJET DNA extraction kit (Thermo Fisher Scientific), following manufacturer's indications.

For routine PCR checking, DNA from *E. coli* was extracted by picking one colony from a LB agar plate, resuspending the colony into 50 µl of molecular grade water (Sigma-Aldrich) and boiling the suspension at 100°C for 5 minutes in a QBD4 heat-block (Grant). The boiled mix was centrifuged on a bench top centrifuge (Eppendorf) to remove bacterial debris at 13,000 rpm for 2 minutes and supernatant was used for PCR (3 µl per reaction) as a template DNA.

2.3.3.3. Mammalian DNA extraction

Attached HeLa cells to the tissue culture dish (Sarstedt) were washed once with Dulbecco's PBS and collected by using a cells scraper (Fisher Scientific). Total DNA was then extracted from HeLa cells by using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich), following manufacturer's instructions.

2.3.4. Molecular cloning and DNA manipulation

As described in figure 2.2, our cloning strategy includes the amplification of the gene of interest by PCR – to generate a transcriptional fusion with a fluorescent protein –, cloning the PCR product into pCRII- TOPO plasmid (4 kb; Thermo Fisher Scientific) in the first place and eventually, cloning the PCR product into P12-MMP (8 kb), a plasmid that was kindly supplied by Dr Felipe X. Pimentel Muiños (Centro de Investigación del Cancer de Salamanca, Spain). The expression of any gene in P12-MMP is driven by the LTR region of the retrovirus. In addition, our P12-MMP vector carries the resistance genes to puromycin and ampicillin, which are selective markers in mammalian cells and bacteria, respectively.

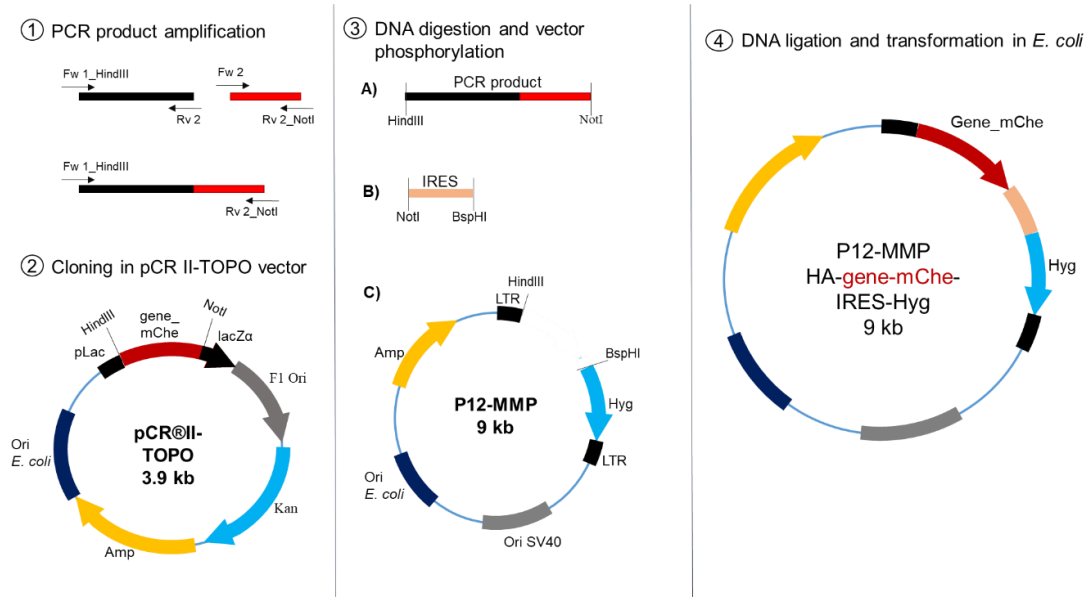

Figure 2.2. The cloning strategy followed to produce constructs that were fused with fluorescent markers. The gene of interest was fused with the fluorescent marker by PCR and cloned into pCRII_TOPO vector (1). Our construct was then double digested from pCRII-TOPO vector using HindIII and NotI as restriction enzymes (2). In parallel, IRES fragment was digested from P12-MMP vector using the restriction enzymes NotI and BspHI and P12-MMP was double digested with HindIII and BspHI enzymes and dephosphorylated (3). Triple ligation was performed and transformed into DH5-α E. coli competent cells (4).

2.3.4.1.pCRII-TOPO cloning

The pCRII-TOPO vector and all the reagents necessary for TOPO cloning were purchased from Thermo Fisher Scientific. Direct cloning of DNA products amplified by proofreading polymerases such as Phusion polymerase is normally difficult since proofreading polymerases remove the 3’ A-overhangs that are necessary for pCRII-TOPO cloning. Therefore, an additional step to clone these blunt-ended fragments was required. Eppendorf tubes containing the PCR products amplified by a proofreading polymerase were placed on ice and 1 unit of 5Prime Hot Master mix polymerase (QuantaBio) was added per tube, without changing the buffer. PCR mixture was incubated at 72°C in the QBD4 heat-block (Grant) for 10

minutes, placed them back on ice and immediately used for pCRII-TOPO cloning reaction.

The pCRII-TOPO cloning reaction was prepared as follows: 4 µl of PCR product, 1 µl of salt solution and 1 µl of TOPO vector. The cloning reaction was mixed, incubated for 30 minutes at room temperature and placed it on ice to proceed to bacterial transformation.

2.3.4.2. Bacterial transformation

E. coli DH5-α-Select Gold (Bioline) were used as chemical competent cells and transformation was performed by heat-shock. 5 µl of DNA were added to a vial with 50 µl of competent cells and the tube was placed on ice for 30 minutes, followed by 30 seconds at 42°C in the QBD4 heat-block (Grant) and returned back to the ice box for 5 last minutes. After the heat-shock was done, 4 volumes of SOC medium (Thermo Fisher Scientific) were added to the mixture and incubated for at least 1 hour at 37°C and with shaking (300 rpm). 50 and 200 µl of the mixture were plated in two LB agar plates, containing 100 µg/ml of ampicillin and plates were incubated at 37°C overnight. Next day, plates were checked, and individual colonies were picked and streaked into new LB agar plates to further validate whether the construction was correct by PCR.

2.3.4.3. DNA digestion

The incorporation of restriction enzyme´s sites were designed using the Addgene's sequence analyser webpage (https://www.addgene.org/analyze-sequence/) and they were purchased from New England Biolabs (NEB). The enzymes employed in this

study were high fidelity (HF) and all of them were sharing the reaction buffer (CutSmart buffer; NEB) to facilitate double digestions.

10 µl of digestion mix was performed as follows: 1 µg of DNA, 1 unit of each restriction enzyme, 1 µl of CutSmart buffer and molecular-grade water up to 10 µl. Digestions were incubated for 30 minutes at 37°C and results of the digestion were visualized by electrophoresis gel (Material and Methods, section 2.3.2).

2.3.4.4. Dephosphorylation of P12-MMP vector

Once the P12-MMP vector has been double digested with the appropriate restriction enzymes, 1 µl of Fast Thermosensitive Alkaline Phosphatase (AP; Thermo Fisher Scientific) was added per 20 µl of final volume. Reaction was mixed by pipetting and incubated for 10 minutes at 37°C. Enzymatic and alkaline phosphatase reactions were stopped by incubating the mixture at 80°C, for 20 minutes. 5 µl of digested and dephosphorylated vector was run in an electrophoresis gel to verify that the digestion was efficient.

2.3.4.5. DNA ligation

The DNA ligation mixture was prepared as follows: 50 ng of dephosphorylated P12-MMP vector, 150 ng of each digested inserts, 1 µl of 10x T4 DNA ligase buffer (New England Biolabs) and 1U of T4 DNA ligase (New England Biolabs) and water up to 10 µl. Reaction was mixed by pipetting and incubated at 16°C in the Thermal Cycler overnight. 5 µl of ligation mixture was used for bacterial transformation of 50 µl of chemically competent cells, as previously described (Material and Methods, section 2.3.4.2).

2.3.4.6. Retroviral production and HeLa transduction

HEK-293T cells were seeded in a 24-wells plate at a cell density of $1x10^5$ cells per well and incubated overnight (37ºC, 5% CO_2). Next day, 1 μg of DNA for transfection was prepared as follows: 0.5 μg of P12-MMP vector with the gene of interest, 0.36 μg of OGP helper plasmid and 0.14 μg of VSV-G helper plasmid. 2 μl of JetPEI (Polyplus) were used to enhance the transfection per μg of DNA and both DNA and JetPEI were diluted in 150mM NaCl to a final volume of 50 μl. JetPEI solution was added to the DNA solution and the mix was immediately vortexed and incubated for 30 minutes at room temperature. Then, 100 μl of jetPEI/DNA mix was added per well to the cells and plates were placed them back into the incubator until the next day. The following day, medium of transfected HEK-293T cells was replaced with fresh complete DMEM and cells were returned to the incubator for another day. After this overnight incubation, supernatant of 293T containing the retroviral particles was collected and filtered with a sterile PES Syringe Filter of 0.45 μm (GE Healthcare Life Sciences) to eliminate HEK-293T cells. Transduction of mammalian cells was carried out as described before (Material and Methods, section 2.2.2), using puromycin (1 μg/ml; Sigma-Aldrich) as selective pressure.

Table 2.6. List of plasmids used in this study

Plasmids	Description	Antibiotic	Source
pCR 2.1-TOPO	Plasmid used for routinely cloning.	Amp (100 µg/ml)	Invitrogen
pCMV-Sport 6	Plasmid carrying the human gene TRAM2.	Amp (50 µg/ml)	Source Bioscience
pLVTHM-Pm_YFPCWT	Plasmid expressing the YFP-CWT phagosomal escape marker.	Amp (50 µg/ml)	Dr. Martin Fraunholz (Giese *et al.*, 2011)
P12-MMP-mCherry-LC3A-IRES-Hygro	P12-MMP vector expressing mChe-LC3A driven by LTR region of the retrovirus.	Amp (100 µg/ml)* Hyg (200 µg/ml)**	Dr. Felipe X. Pimentel Muiños (Boada-Romero *et al.*, 2013)

*Antibiotic resistance in bacterial cells
**Antibiotic resistance in eukaryotic cells

2.3.5. ShRNA screening

For shRNA screening, PCRs were performed over genomic DNA samples employing oligonucleotides complementary to constant sequences in all shRNA vectors (Lentiplex Amplification Primers) and following the protocol described before (Material and Methods, section 2.3.1). The individual shRNA occurrence in the different samples was provided by the company Sigma-Aldrich. Briefly, genomic DNA samples were submitted to the Sigma Deconvolution platform, and the abundance of each shRNA clone in samples was tested by amplifying and next-generation sequencing (at ×1,000) shRNA regions and barcoding samples; short reads were aligned to the reference. Data were obtained as the number of shRNA sequences per clone per sample, as previously described (Gawrzak *et al.*, 2018).

2.4. IMMUNOPROTEOMICS

2.4.1. Cell extractions and protein lysates

Cells were harvested by adding ice-cold Dulbecco's PBS to each well and detached by using a cell scrapper (Fisher Scientific). Cell suspensions were transferred to a 15 ml Falcon tube and centrifuged (2,000 rpm, 10 min, 4°C). Cells pellet was washed with ice-cold Dulbecco's PBS and immediately placed on ice for cell lysis. Lysis buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 5 mM EDTA; 1% NP40) was reconstituted with protease inhibitor cocktail (Sigma-Aldrich), phosphatase inhibitor cocktail (Sigma-Aldrich) and phenylmethylsulfonyl fluoride (PMSF;Sigma-Aldrich), following manufacturer's instructions. Cell pellets were

resuspended in 50 µl of lysis buffer per well, transferred to Eppendorf's tubes and incubated on ice for 30 minutes, applying heavy vortexing every 10 minutes. Then, samples were centrifuged at maximum speed for 5 minutes at 4°C and supernatants were transferred to new Eppendorf´s tubes and immediately stored at -80ºC until needed.

Protein concentration was quantified by using DC Protein Assay Kit (Biorad) and every cell lysate was adjusted to same concentration by diluting each sample in lysis buffer containing protease and phosphatase inhibitors.

2.4.2. SDS-PAGE electrophoresis

Cell extractions were mixed with 2X Reducing Sample Buffer (100 mM Tris, pH 6.8; 10% β-mercaptoethanol; 4% SDS; 0.3% bromophenol blue; 20% glycerol) and boiled in a QBD4 heat block (Grant) at 100ºC for 5 minutes. Samples were loaded on polyacrylamide gels using 1X of Running Buffer (25 mM Tris; 20 mM Glycine; 0.05% SDS) and run on a Mini-PROTEAN Tetra electrophoresis system (Bio-Rad) with a constant voltage of 100 V for 90 minutes using a PowerPac Basic Power Supply (Bio-Rad).

2.4.3. Western-blotting and antibody detection

Proteins were transferred onto nitrocellulose membranes (GE Healthcare Life Sciences) by using a Mini Trans-Blot cell (Bio-Rad) with 1X Transfer buffer (25 mM Tris; 192 mM Glycine; 20% methanol) and Whatman filter paper (GE Healthcare Life Sciences), following manufacturer's guidelines. The Western-blot

was run on the Tetra electrophoresis system (Bio-Rad) at a constant voltage of 90 V for two hours.

Nitrocellulose membranes were blocked using 5% skimmed milk diluted in Tris buffer saline (TBS) solution (50 mM Tris, pH 7.6; 150 mM NaCl) for 1 hour at room temperature. Once the blocking solution was removed, membrane was washed twice with TBS containing 0.05% of Tween20 (TBS-T) and subsequently incubated overnight at 4°C with the primary antibody, diluted in TBS-T containing 2% BSA and 0.1% sodium azide. After three washes of TBS -T, membrane was incubated with the corresponding secondary antibody diluted in 5% skimmed milk/TBS-T for 1 hour at room temperature. Membrane was again washed three times with TBS-T and was developed employing Odyssey® Fc Imaging System (Licor). Images were taken, processed and quantified by using Image Studio Software (Licor).

Primary and secondary antibodies used in this study are listed in table 2.7 and dilution recommended by the suppliers and used for the experiments is also specified in table 2.7.

Table 2.7. List of antibodies used in this study

	Species	Type	Dilution	Source	Purpose
Primary antibodies					
β-actin	Mouse	Monoclonal	1:1000	Sigma-Aldrich	Western-blot
GAPDH	Mouse	Monoclonal	1:500	Santa Cruz Biotechnology	Western-blot
TRAM2	Rabbit	Polyclonal	1:1000	Novus biologicals	Western-blot
LC3B	Rabbit	Polyclonal	1:2000	Thermo Scientific	Western-blot
AMPK	Rabbit	Polyclonal	1:1000	Cell signalling	Western-blot
p-AMPK	Rabbit	Polyclonal	1:1000	Cell signalling	Western-blot
ERK 1/2	Rabbit	Polyclonal	1:1000	Cell signalling	Western-blot
pThr202/Tyr204-ERK 1/2	Mouse	Polyclonal	1:1000	Cell signalling	Western-blot
N-WASP	Mouse	Polyclonal	1:1000	Novus biologicals	Western-blot
pTyr256-N-WASP	Rabbit	Polyclonal	1:1000	Novus biologicals	Western-blot
EPHA2	Mouse	Monoclonal	1:100	Santa Cruz Biotechnology	Western-blot
pSer901-EPHA2	Rabbit	Polyclonal	1:1000	Signalway Antibodies	Western-blot
c-Jun	Rabbit	Polyclonal	1:1000	St John's Laboratory	Western-blot
pTyr231-c-Jun	Rabbit	Polyclonal	1:1000	St John's Laboratory	Western-blot
p-Tyr239-c-Jun	Rabbit	Polyclonal	1:1000	St John's Laboratory	Western-blot
S. aureus Methicillin Resistant	Mouse	Polyclonal	1:2000	Thermo Scientific	Immunofluorescence
Secondary antibodies					
IRDye® 680LT Goat anti-mouse	Mouse		1:5000	Licor	Western-blot
IRDye® 800LT Goat anti-rabbit	Rabbit		1:5000	Licor	Western-blot
Donkey anti-mouse TRITC conjugate	Mouse		1:5000	Thermo Scientific	Immunofluorescence

2.5. PHOSPHOPROTEOMICS

2.5.1. Samples preparation for phosphoproteomics analyses

After 6 hours of MRSA infection, cell extracts were obtained by lysing the cells with 50 µl of lysis buffer (20mM HEPES (pH 8) buffer containing 8M urea, 1mM Na_3VO_4, 1mM NaF, 1mM β-glycerol phosphate and 2.5mM $Na_2H_2P_2O_7$) per well. After lysis buffer was added to the cells, samples were thoroughly vortexed for 5 minutes and immediately stored at -80ºC. Following sonication, cell lysates were centrifuged at 20,000 g for 10 min and the protein concentration was determined by Bradford analysis.

Protein digestion was performed at Barts Cancer Institute facilities. A digestion protocol was applied as follows: cell extracts were diluted to 2M Urea with 20mM HEPES pH 8.0 and incubated with immobilized TLCK-trypsin (20 TAME units/mg) for 16 h at 37ºC. Digestion was then stopped by adding 1% of trifluoroacetic acid (TFA). The resultant peptide solutions were desalted using Sep-Pak C18 columns (Waters UK Ltd, Manchester, UK) following manufacture's guidelines (Casado and Cutillas, 2011).

2.5.2. LC-MS/MS

Mass Spectrometry analyses were also carried out at Barts Cancer Institute facilities. LC-MS/MS protocol was applied as follows: phosphopeptide enrichment was performed using an Immobilized Metal Ion Affinity Chromatography (IMAC) enrichment protocol as previously described (Alcolea, Kleiner and Cutillas, 2009). Phosphopeptides were detected in a Liquid Chromatography Tandem Mass

Spectrometry (LC-MS/MS) system as previously reported (Casado and Cutillas, 2011; Alcolea *et al.*, 2012).

Mascot Daemon (v2.2.2; Matrix Science, London, UK) was employed to analyse the LC-MS/MS data. The parameters included trypsin as digestion enzyme, carbamidomethyl (C) as fixed modification and pyro-glu (N-terminal), oxidation (M) and phosphor (STY) as variable modifications. Datasets were searched with a mass tolerance of ±7 ppm and a fragment mass tolerance of ±600 mmu. Hits were considered significant when they had an expectation value <0.5 (as returned by Mascot). False discovery rates were ~2% as determined by decoy database searches. An bespoke script was used to extract Mascot results, which were then placed in Excel file database for further analysis (Montoya *et al.*, 2011). PESCAL was used to quantify the intensities of peptides present in the database across all samples to compare. PESCAL uses the restrictions on the molecular mass, retention time, charge and isotopes distribution to confidently identify the studied phosphopeptides (Cutillas and Vanhaesebroeck, 2007). The resulting quantitative results were then exported to Excel files for further normalization and statistical analysis. Peptides intensities were normalized to the total chromatogram intensity and to the mean intensities across samples to be compared (Alcolea *et al.*, 2012).

2.5.3. Functional analysis

Phosphoproteomics data was plotted as heat-map as well as Volcano plot. Statistical differences in phosphopeptides quantification were assessed by Student´s t-test and adjusted for multiple testing using the Benjamini Hochberg false discovery rate method. R software was used for principal component analysis (PCA) and

performed by Pedro R. Cutillas. Regarding functional analysis, DAVID Bioinformatic software v.6.8 (Huang, Sherman and Lempicki, 2009) was used to identify the most important KEGG pathways, and KSEA analysis (performed by Pedro R. Cutillas as described in Casado et al. 2013) were carried out to find out the kinase signalling pathways that were activated under Ibrutinib treatment.

2.6. METABOLOMICS

2.6.1. Samples preparation for extracellular metabolome analysis

After 6 hours of infection, 1.5 ml of medium was sampled, centrifuged (2,000 rpm, 5 min, 4ºC) and the supernatant was stored immediately at -80ºC. To prepare samples for analysis by Nuclear Magnetic Resonance (NMR) spectroscopy, 0.8 ml of cell supernatant were mixed with 0.2 ml of NMR buffer. The NMR buffer contained 1 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as internal standard and 25 mM NaN_3 (to ensure sterility) in ^2H_2O that serves as frequency lock for the spectrometer, resulting in a final concentration 0.2 and 5 mM, respectively.

2.6.2. Nuclear Magnetic Resonance (NMR)

NMR spectroscopy was performed on a Bruker DRX600 Avance spectrometer (Bruker Biospin, Rheinstetten, Germany) with a magnetic field strength of 9.4T and 400 MHz ^1H resonance frequency. Spectra (at 298K) were acquired into 32K data points using a one-dimensional solvent-suppression sequence, essentially as

described before (Beckonert *et al.*, 2007). Data analysis was performed using in-house software, following a work-flow described in La Rosa et al. 2015.

2.6.3. Samples preparation for intracellular metabolome analysis

Cells were washed with 1 ml of ice-cold Ringer´s solution (Oxoid) after 6 hours of infection and were quenched by adding 1 ml of cold (-20ºC) LC-MS grade methanol (Sigma-Aldrich) (Sellick *et al.*, 2009). Cells were then detached by using a cell scraper (Fisher Scientific) and the cold methanol suspension was transferred into a clean Eppendorf tube. Extraction was repeated with a further 0.5 ml of cold methanol and extracts were pooled and stored at -80ºC.

To obtain separated organic and aqueous fractions, extracts were dried using an Eppendorf Vacufuge Concentrator (Eppendorf). A dual phase extraction was performed by adding 300 µl of $CHCl_3$/MeOH (2:1; Sigma-Aldrich) and vortexing for 30 seconds. After addition of 300 µl of water and centrifugation (13,000 rpm, 10 minutes, RT), the top aqueous layer was transferred into an inactivated glass vial (Agilent Technologies) and dried before storing at -80ºC. The lower organic layers were placed into glass vials and dried overnight before being stored at -80C. The process was repeated, and fractions were pooled.

For Gas Chromatography-Mass Spectrometry (GC−MS) analysis, aqueous fractions were derivatized by a two-step methoximation/silylation derivatization procedure. For normalisation, a standard of 10 µl of 1.5 mg/ml myristic acid d27 standard solution (Sigma-Aldrich) was added and samples were dried. The dried samples were first methoximated using 20 µl of 20 mg/mL methoxyamine hydrochloride (Sigma-Aldrich) in anhydrous pyridine at 37 °C for 90 min. For

unlabelled samples, this was followed by silylation with 80 µl of N-methy-N-(trimethylsilyl) trifluoroacetamide (MSTFA; Thermo Fisher Scientific) at 37 °C for 30 min (Kind *et al.*, 2009). Labelled-samples were derivatized by adding 80 µl of N-(tert-butyldimethylsilyl)-N-methyltrifluro-acetamide (MBTSFA, Thermo Fisher Scientific). After being vortexed, samples were placed in a heat block (Grant) at 70°C for 1 hour. Eventually, samples were centrifuged at 2,000 rpm for 5 minutes prior to transferring them into a clean vial for GC-MS analysis.

The organic fraction was reconstituted in 300 µl of methanol/toluene solution (1:1 ratio), treated with 200 µl of 0.5 M sodium methoxide and incubated for 1 hour at room temperature. Reaction was stopped by adding 500 µl of 1M NaCl and 25 µl of concentrated HCl. Fatty acids were extracted by using 500 µl of hexane and organic layers were dried in the fume cupboard under N_2. Organic samples were then derivatized with 40 µl acetonitrile and 40 µl of MBTSFA (Thermo Fisher Scientific) and incubated at 70°C for 1 hour. Samples were finally centrifuged at 2,000 rpm for 5 minutes prior to transferring them into a clean vial for GC-MS analysis.

2.6.4. Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analysis was performed on an Agilent 7890 GC equipped with a 30 m DB-5MS capillary column with a 10 m Duraguard column connected to an Agilent 5975 MSD operating under electron impact (EI) ionization (Agilent Technologies). Samples were injected with an Agilent 7693 autosampler injector into deactivated splitless liners according to the method of Kind et al. (2009), using helium as the carrier gas (Tredwell and Keun, 2015).

Metabolites in the unlabelled pool were identified and quantified using a workflow described in Behrends et al. 2011. Briefly, samples were deconvoluted in AMDIS (Stein, 1999; Kind *et al.*, 2009) and quantified using an in-house script. Integration of labelled metabolites was carried out based on an in-house fragment/retention time database using an updated version of the Matlab script capable of natural isotope correction (Behrends, Tredwell and Bundy, 2011; Tredwell and Keun, 2015).

2.7. MICROSCOPY

2.7.1. Immunofluorescence microscopy

HeLa cell lines expressing the protein/s of interest fused with a fluorescent marker were seeded in coverslips at a cell density of $7.5x10^4$ cells per well in 24 well plates and *S. aureus* USA300-GFP (Reichmann *et al.*, 2014) was used to infect the cells at a MOI of 100 (Table 2.3). Infection was performed as previously described (Material and Methods, section 2.2.3) and after 6 hours of infection, cells were fixed with 0.5 ml of fresh 4% of paraformaldehyde (PFA; Fisher Scientific) for 15 minutes. Coverslips were then washed twice with PBS and mounted on microscope slides (Thermo Fisher Scientific) using ProLong Gold Antifade mountant with DAPI (Thermo Fisher Scientific) for nuclear staining.

For routine microscopy assays, the Olympus BX51 immunofluorescence microscope was used to observe the preparations and pictures were taken using Pixera software.

2.7.2. Confocal microscopy

Slides were observed on a confocal microscope ZEISS LSM 800 with Airyscan and images were acquired and processed by using Zen Blue Software (Zeiss).

2.7.2.1. Phagosomal escape quantification by confocal microscopy

HeLa cells expressing the phagosomal escape reporter mCherry-CWT (Grosz, Kolter, Paprotka, A. C. Winkler, *et al.*, 2014) were seeded on coverslips in 24-well plates ($7x10^4$ cells per well) and infected with *S. aureus* USA300-GFP (Reichmann *et al.*, 2014) at a MOI of 100. After 6 hours of infection, medium was aspirated, and cells were fixed and analysed as previously described.

2.8. STATISTICAL ANALYSIS

Statistical tests and graphs plotting were conducted using GraphPad Prism software.

Student's t-test and *post hoc* Tukey's multiple comparison tests were employed to examine significant differences across treatments.

3. Intracellular *Staphylococcus aureus* modulates host central metabolism to activate autophagy

3.1. BACKGROUND

Bacterial pathogens requires large amount of energy to support intracellular proliferation and hence, the interaction between intracellular pathogens and host cell metabolism is an important feature of virulence, since bacteria need to adapt to the host microenvironment (Abu Kwaik & Bumann 2015; Dandekar & Eisenreich 2015). Once bacteria have been internalized, both bacteria and the host cell share - and compete for - the same nutrients, such as carbon sources or amino acids (Abu Kwaik & Bumann, 2015). In addition, metabolic products of the pathogen, e.g. derived from fermentation, are released to the host cell where they can activate different responses and alter the host metabolism (Olive and Sassetti, 2016). This interplay between host and pathogen represents a complex biological process and the site of infection could be considered as a closed system where the metabolism of two organisms work in parallel (Olive and Sassetti, 2016). The manipulation of

specific bacterial adaptations as well as the host-metabolic pathways targeted by the pathogen may be important to develop future treatments against bacterial infection. That said, the knowledge in this area is still limited (Dandekar & Eisenreich 2015).

Pathogens exploit the host cell as a rich source of nutrients and in response, host immune responses seek to restrict bacterial access. Consequently, bacterial pathogens have developed different mechanisms to evade host immune response and virulence factors to access nutrients from the host (Abu Kwaik and Bumann, 2015).

Host immune responses are commonly activated by microbial surface components such as lipopolysaccharides (LPS) and other pathogen associated molecular patterns (PAMPs) molecules (Eisenreich *et al.*, 2015) leading to several host immune responses. Some of these responses have a direct antimicrobial activity such as the production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) (Nathan and Shiloh, 2000). Others seek to deprive the pathogen from the access to scarce and important nutrients: expression of the transporter Nramp1 promoting iron depletion (Cellier, Courville and Campion, 2007) or the activation of indoleamine 2,3-dioxygenase by IFN-γ to degrade tryptophan (Taylor and Feng, 1991).

As a result, successful intracellular pathogens must overcome these antimicrobial host responses and have developed different mechanisms to acquire nutrients from the host (Abu Kwaik and Bumann, 2015). Nutrients acquisition can be achieved by different actions: by altering host metabolic pathways (Escoll *et al.*, 2017), by raising nutrient import (Lee *et al.*, 2009) or by exploiting/subverting host

mechanisms to degrade macromolecules such as autophagy (Steele, Brunton and Kawula, 2015).

Autophagy is a catabolic mechanism that involves the formation of double-membrane vesicles – autophagosomes – and subsequent lysosomal fusion to degrade damaged or undesirable cytosolic material (Mizushima, 2007; Glick, Barth and Macleod, 2010). It is a well-conserved pathway in eukaryotic cells and plays important physiological roles in response to nutrient starvation, physiological stress and recycling of organelles (Mizushima, 2009; Glick, Barth and Macleod, 2010; Galluzzi *et al.*, 2014).

Despite the names, (auto)-phagosomes, these structures are also involved in a common host response against intracellular bacteria called xenophagy (Wileman, 2013). It is known that several intracellular pathogens, including *S. aureus*, can subvert xenophagy to persist within the autophagosomes before escaping to the cytosol (Schnaith *et al.*, 2007). However, it is not yet fully understood how *S. aureus* achieves this and which host pathways and/or metabolites it uses to enhance its intracellular survival and/or replication. *S. aureus* may also exploit this host pathway to support its energy demands since autophagy degrades macromolecules into accessible nutrients (Steele, Brunton and Kawula, 2015).

Although there is still limited knowledge of how the host metabolism is triggered by different pathogens, the research that has been done on this topic was focused on more established intracellular pathogens such as *S. flexneri*, *E. coli*, *L. monocytogenes*, *S.* Typhimurium, *L. pneumoph*ila and *C. trachomatis* (Eisenreich *et al.*, 2015). By contrast, not a lot is known about how the host cell metabolism is modulated and exploited by the pathogen *S. aureus*.

The aim for this chapter was to understand the host metabolic changes induced by intracellular MRSA infection with a view of identifying possible novel host-directed anti-infective strategies against MRSA infections.

3.2. RESULTS

3.2.1. The extracellular metabolome of MRSA-infected host cells

We used nuclear magnetic resonance (NMR) spectroscopy to analyse the media composition during gentamycin-protection assays in HeLa cells exposed to both alive and heat-killed *S. aureus* USA300. Results showed an increased concentration of organic acids in the medium of infected cells with viable USA300, such as formic, pyruvic and succinic acid. In particular, the highest increase was detected for acetic acid, with an increase of more than 100-fold compared to uninfected cells (Fig. 3.1). Levels of some amino acids (leucine, alanine and methionine) were also increased in the medium of infected cells (Fig. 3.1). In contrast, glucose levels were significantly reduced when compared to the uninfected control (Fig. 3.1), suggesting more rapid glucose uptake. No significant changes were found in the medium of cells infected with heat-killed USA300, suggesting that the increased secretion of organic acids and the lower levels of glucose were resulting from metabolic changes in the host cell triggered by viable intracellular bacteria.

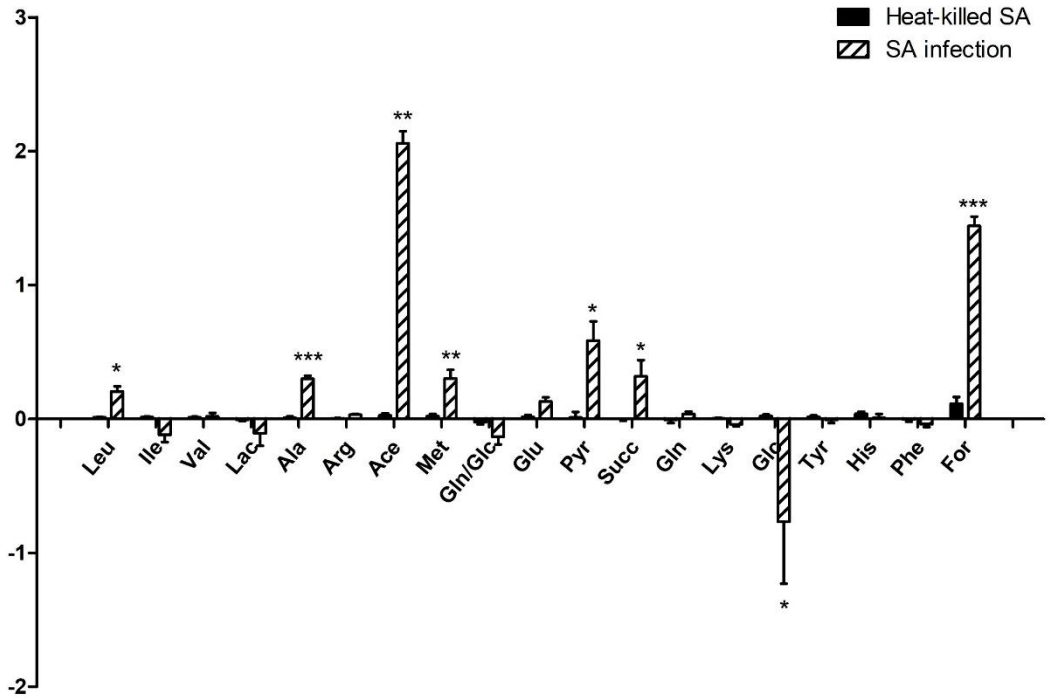

Figure 3.1. Comparison of metabolites identified in the media after MRSA infection. HeLa cells were exposed to alive and heat-killed USA300 (MOI 100; 6 hours) and media composition was analysed by NMR spectroscopy. Results are shown as log10 fold change relative to the uninfected condition and the figure shows mean ± SE of two independent experiments performed in triplicates. Shredded bars and black bars represents cells infected with USA300 and heat-killed USA300, respectively. p-value ≤0.05 (*); ≤0.01 (**); ≤0.001 (***). Leu: leucine; Ile: isoleucine; Val: valine; Lac: lactate; Ala: alanine; Arg: arginine; Ace: acetate; Met: methionine; Glu: glutamate; Pyr: pyruvate; Succ: succinate; Gln: glutamine; Lys: lysine; Glc: glucose; Tyr: tyrosine; His: histidine; Phe: phenylalanine; For: formate.

3.2.2. Changes in the host cell metabolism in response to MRSA infection

3.2.2.1. Central carbon metabolism after *S. aureus* USA300 infection

The intracellular metabolome of MRSA-infected cells was analysed by GC-MS (Figs. 3.2 & 3.3). We detected a major increase of several glycolytic intermediates during MRSA invasion. Levels of both 3-phosphoglycerate (3-PG) and phosphoenolpyruvate (PEP) were over 50 times higher in MRSA infected cells when compared to uninfected cells or cells exposed to heat-killed bacteria (Fig.

3.2). In addition, the glycerate pool size in the MRSA-infected cells significantly increased when compared to controls (Fig. 3.2). The labelling distribution of glycerate (Fig. 3.3) suggests that it was formed from glycolytic 3-phosphoglycerate. In contrast, pyruvate levels only increased slightly in the MRSA-infected cells, whereas lactate levels remained mostly unchanged among the three conditions (Fig. 3.2).

Several tricarboxylic acid (TCA) cycle intermediates such as α-ketoglutarate, citrate and succinate were present at higher levels in HeLa cells infected with USA300 (Fig. 3.2). Interestingly, this increase was not universal, as levels of fumarate remained unchanged among the three conditions and malate levels were reduced in cells infected with MRSA (Fig. 3.2). Further, stable isotope tracing suggested that most of the carbon of succinate is derived from glutamine; in contrast, the labelling pattern of fumarate and malate showed an enhancement of glucose-derived label, suggesting an increased importance of extra-mitochondrial pools of these metabolites. Interestingly, an increase in glucose-derived carbon can also be seen in aspartate (Fig. 3.3). For both fumarate and aspartate, this increase was due to a rise in the M+3 isotope (Fig. 3.4).

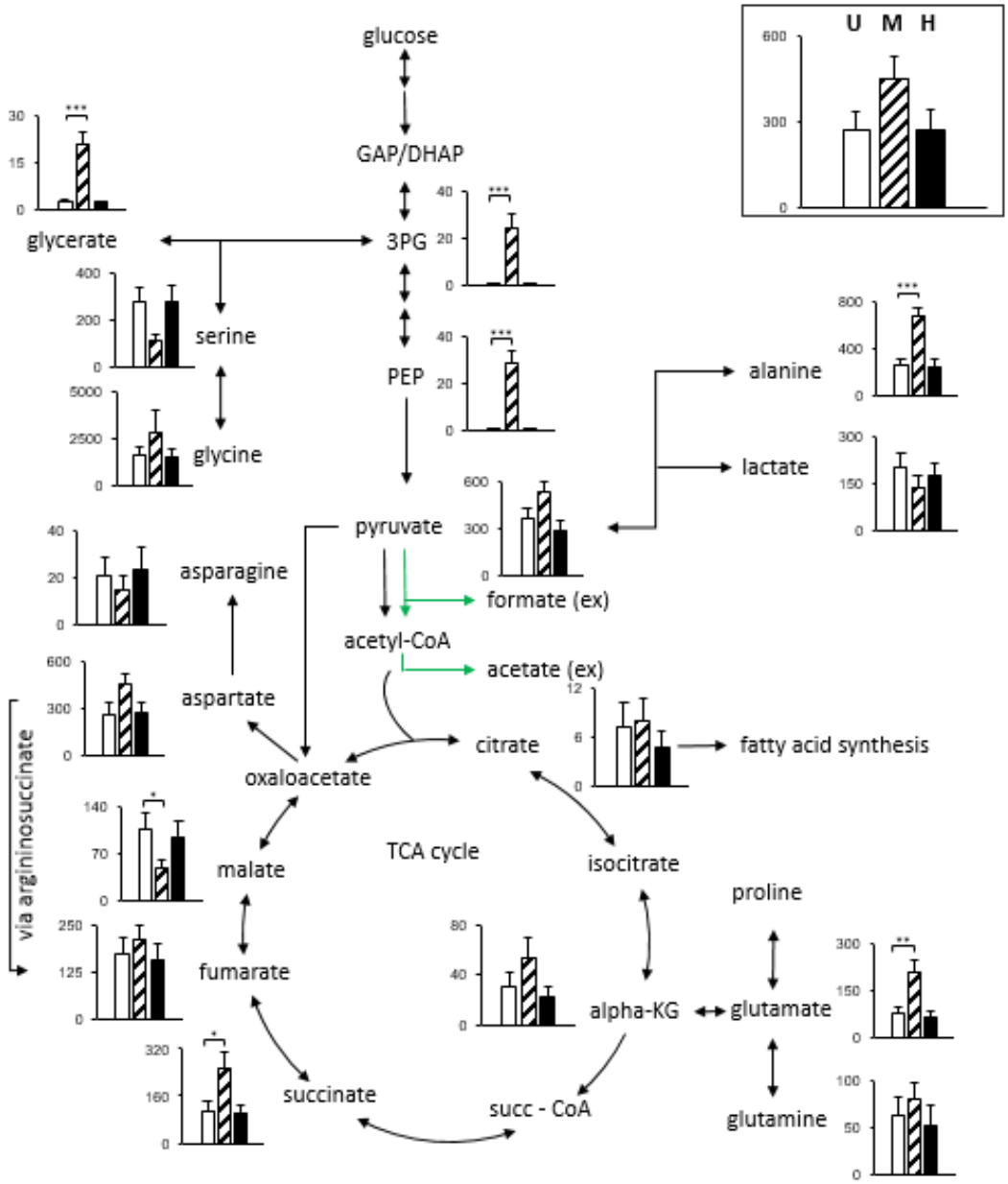

Figure 3.2. Differences in the pool sizes of metabolites of the central carbon metabolism after *S. aureus* USA300 infection in HeLa cells. HeLa cells were lysed after gentamycin-protection assays with both alive and heat-killed USA300 and metabolites were detected by GC-MS. Graphs show absolute levels of each metabolite in uninfected cells (white bars), cells infected with USA300 strain (shaded bars) and cells exposed to heat-killed USA300 strain (black bars). Figure shows means ± SE of two independent experiments performed in triplicates. Statistical differences were tested using Student´s t-test against uninfected cells. p-value ≤0.05 (*); ≤0.01 (**); ≤0.001 (***). Y-axis scale units are arbitrary units (x1,000). Green lines: metabolites of microbial origin.

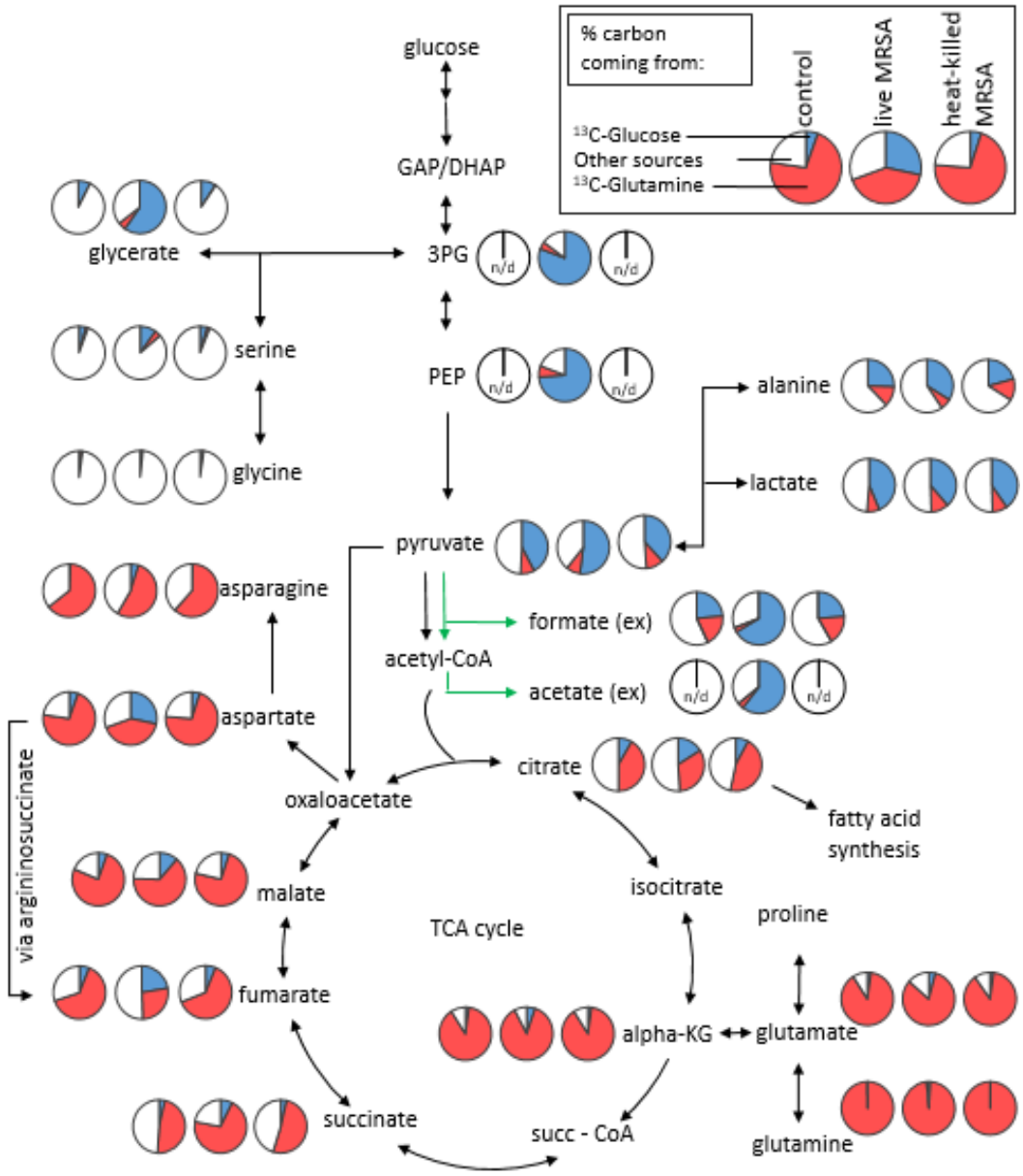

Figure 3.3. Differences in the labelling pattern of metabolites of the central carbon metabolism after MRSA infection. HeLa cells were infected with USA300 and exposed to heat-killed USA300 strains (MOI 100; 6 hours). Metabolites and labelling distribution were obtained by GC-MS. Figure illustrates the labelling pattern of each metabolite in uninfected cells (left pie-chart), cells infected with USA300 strain (middle pie-chart) and cells exposed to heat-killed USA300 strain (right pie-chart). Pie-charts depict the labelling distribution where blue slides represent labelled carbon coming from glucose; red slides are labelled carbon coming from glutamine and white slides show carbon from other sources. Green lines: metabolites of microbial origin.

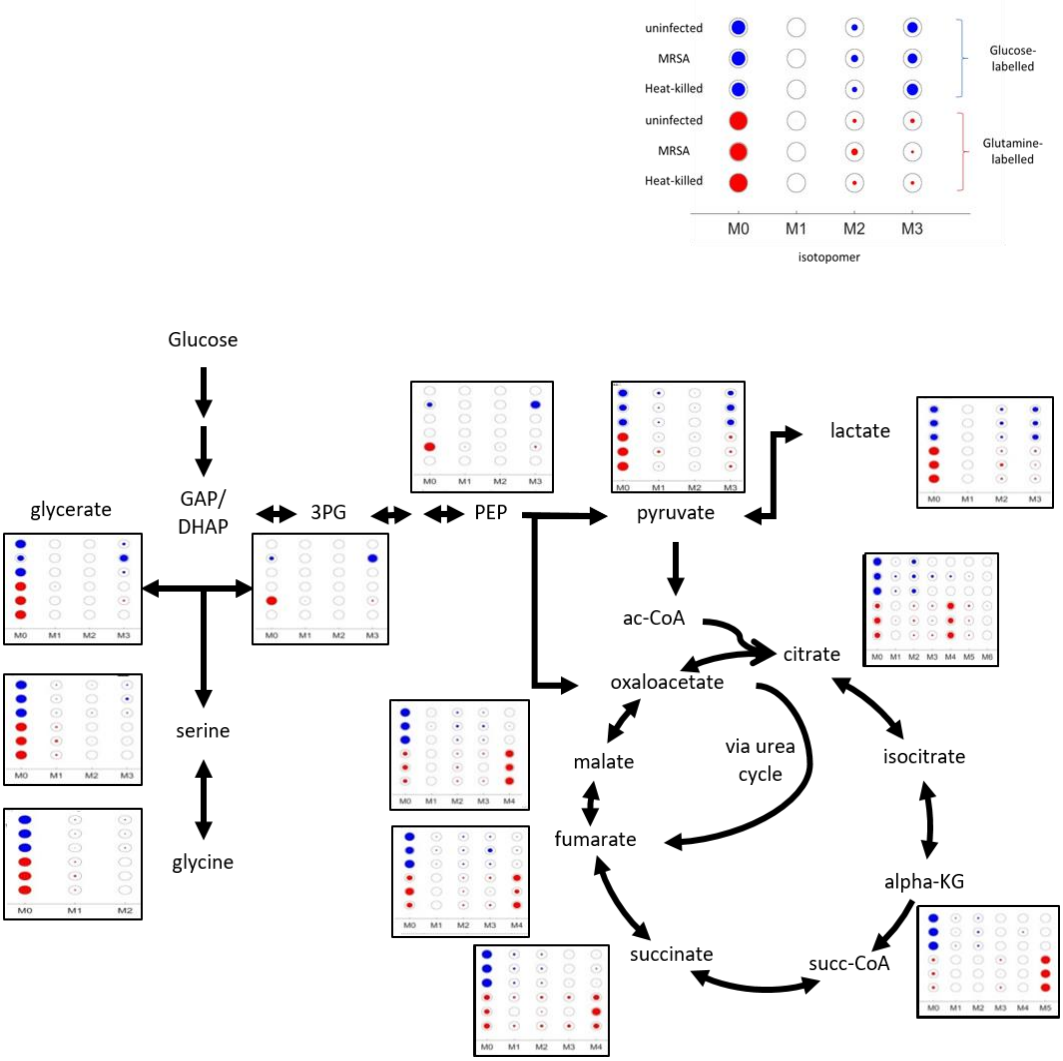

Figure 3.4. Analysis of labelling pattern distribution of metabolites of the central carbon metabolism after MRSA infection. HeLa cells were infected with USA300 (MOI 100) for 6 hours and labelling distribution were obtained by GC-MS. This figure illustrates the labelling pattern of each metabolite in cells infected with USA300 strain. The area of the filled circle depicts the fraction of label for each isolate, where blue represents carbon coming from glucose-labelled, red is carbon coming from glutamine-labelled and the black line is 100% of labelling.

3.2.2.2. Amino acids metabolism

We observed an activation of glutaminolysis in HeLa cells infected with USA300, with higher uptake of glutamine from the medium and unchanged intracellular levels. In contrast, glutamate and alanine levels were significantly higher in MRSA-infected cells compared to uninfected cells or cells exposed to heat-killed MRSA

(Fig. 3.2). In the case of alanine, there is a slight increase of glucose-derived label, indicating increased flux from pyruvate (Figs. 3.2 & 3.3).

Branched-chain amino acids are thought to be important for the virulence of *S. aureus* (Kaiser *et al.*, 2015). However, we have observed no differences in the levels of valine, leucine and isoleucine among the three experimental conditions (Fig. 3.5).

Importantly, the pool sizes of histidine, threonine and serine (three essential amino acids for both *S. aureus* and HeLa cells) were significantly reduced after USA300 infection (Fig. 3.5). In contrast, phenylalanine levels of infected cells were also increased. While phenylalanine cannot be synthesized *de novo* by mammalian cells, MRSA is able to synthesize phenylalanine from phenylpyruvate and histidine via HisC (SAUSA300_0708), a histidinol-phosphate aminotransferase, potentially explaining both higher levels of phenylalanine and lower levels of histidine in MRSA-infected cells.

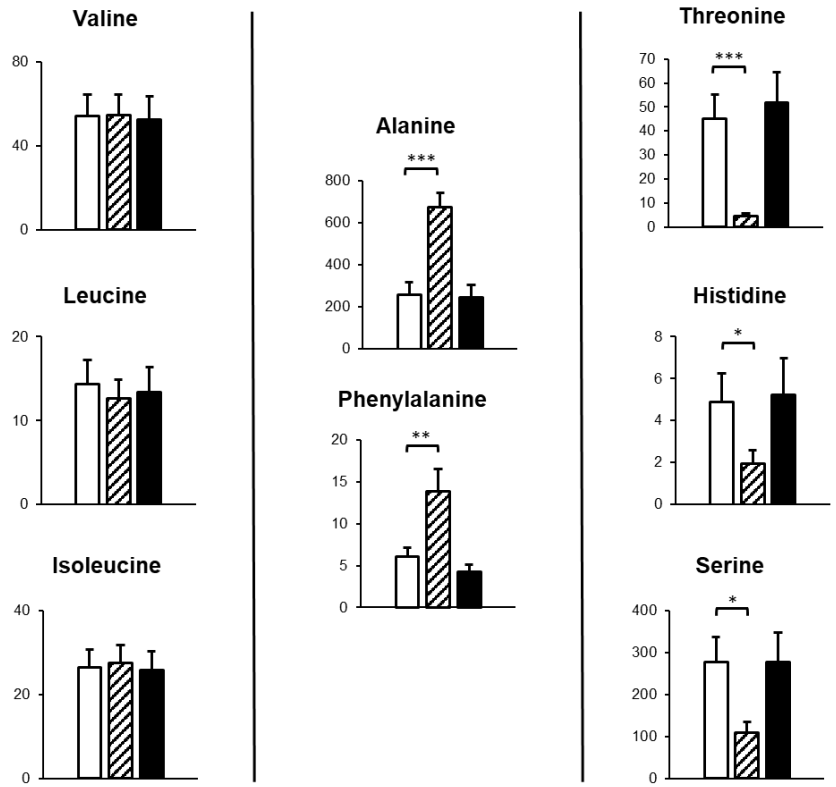

Figure 3.5. Changes of absolute levels of amino acids in HeLa cells after *S. aureus* USA300 infection. HeLa cells were infected with USA300 and exposed to heat-killed USA300 strains (MOI 100; 6 hours). Levels of amino acids were detected and quantified by GC-MS. Graphs show absolute levels of each amino acid in uninfected cells (white bars), cells infected with USA300 strain (shaded bars) and cells exposed to heat-killed *S. aureus* USA300 strain (black bars). Figure shows mean ± SE of two independent experiments performed in triplicates. Statistical differences were tested using Student´s t-test against uninfected cells. p-value ≤0.05 (*); ≤0.01 (**); ≤0.001 (***). Y-axis scale units are arbitrary units (x1,000).

3.2.2.3. Changes in the central carbon metabolism after vancomycin-protection assays with *S. aureus* NCTC 13626

The significance of certain host-pathogen interactions may vary depending on the cell line or bacterial strain tested (Fraunholz and Sinha, 2012; Strobel *et al.*, 2016). For example, the metabolism of cancer cell lines is conditioned by the Warburg effect (Potter, Newport and Morten, 2016) and therefore, the metabolomics findings described in HeLa cells may not be applicable to primary cells. To address this, we studied the metabolism of HeLa cells and BALB/c mouse bone marrow

macrophages when infected with the MRSA strain NCTC 13626, a reference strain for the healthcare-associated MRSA ST239 lineage (Edgeworth *et al.*, 2007; Holden *et al.*, 2010). NCTC 13626 is resistant to gentamycin, therefore the infection assays were carried out in the presence of vancomycin (Fig. 2.1). Interestingly, most of the changes induced in the metabolism of both types of host cells tested with NCTC 13626 were in concordance with the results observed in HeLa cells infected with USA300 (Figs. 3.6 & 3.7). The levels of glucose were reduced in HeLa cells and bone marrow macrophages in response to NCTC 13626 infection (Figs 3.6 & 3.7), whereas glutamate levels were significantly increased, especially in bone marrow macrophages (Fig. 3.7), suggesting an activation of both the glycolysis and glutaminolysis pathways. The increased levels of glycolytic intermediates were found in HeLa cells irrespective of the infecting strain.

Similar to the USA300-infected cells, several metabolites from the TCA cycle such as citrate and α-ketoglutarate were significantly increased in NCTC 13626-infected cells when compared to uninfected controls (Figs 3.6 & 3.7). The levels of succinate were also increased in HeLa cells infected with NCTC 13626 (Fig. 3.6), whereas succinate levels were stable in bone marrow macrophages (Fig. 3.7). In contrast with USA300 infection, both levels of fumarate and malate were higher in HeLa cells and bone marrow macrophages when infected with NCTC 13626, suggesting a strain-dependent metabolic change (Figs 3.6 & 3.7).

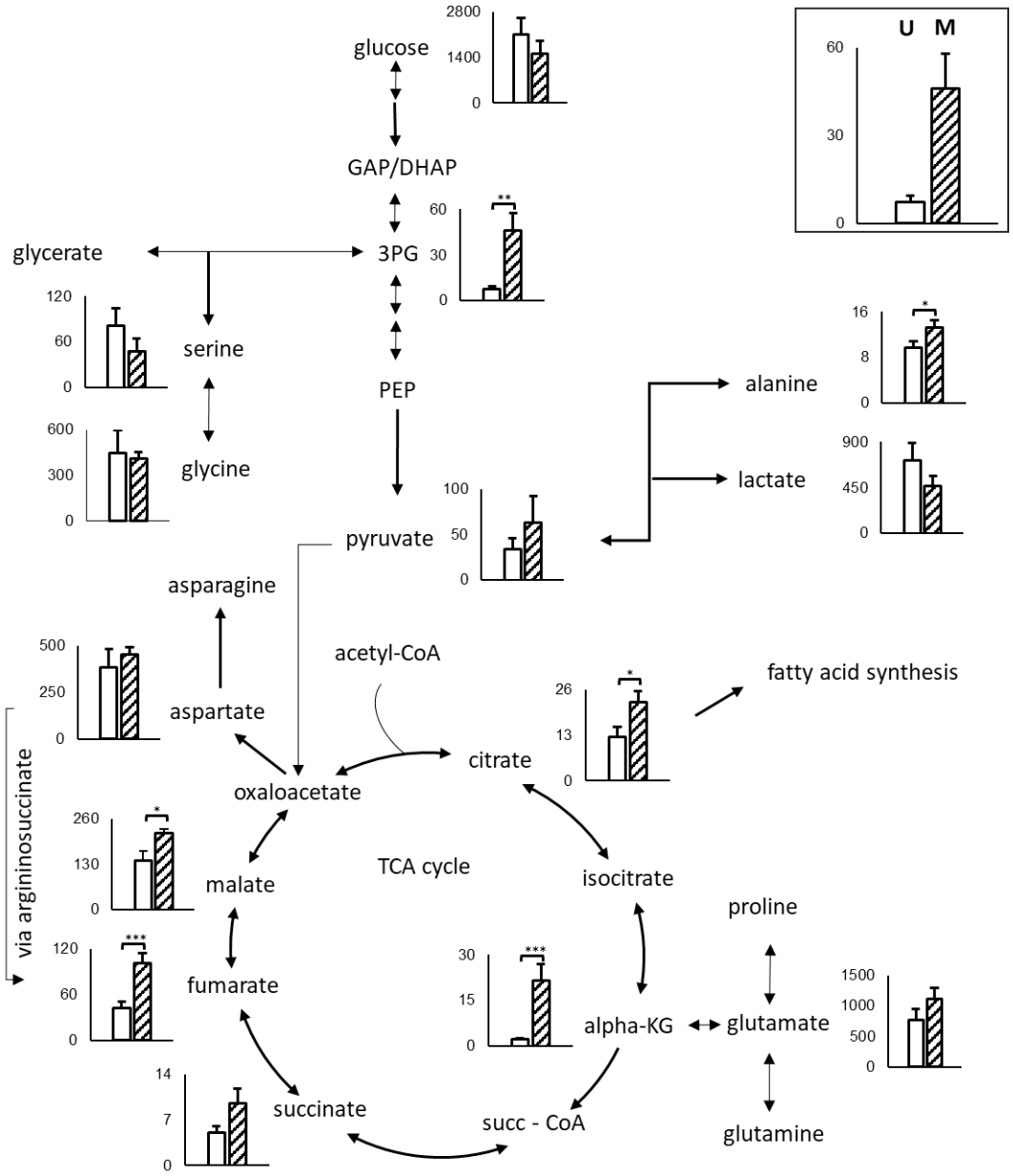

Figure 3.6. Differences in the pool sizes of metabolites of the central carbon metabolism after *S. aureus* NCTC 13626 infection of HeLa cells. HeLa cells were lysed after vancomycin-protection assays with NCTC 13626 (MOI 100; 6 hours) and metabolites were detected by GC-MS. Graphs show absolute levels of each metabolite in uninfected cells (white bars) and cells infected with NCTC 13626 strain (shaded bars). This figure shows means ± SE of two independent experiments performed in triplicates. Statistical differences were tested using Student´s t-test against uninfected cells. p-value ≤0.05 (*); ≤0.01 (**); ≤0.001 (***). Y-axis scale units are arbitrary units (x1,000).

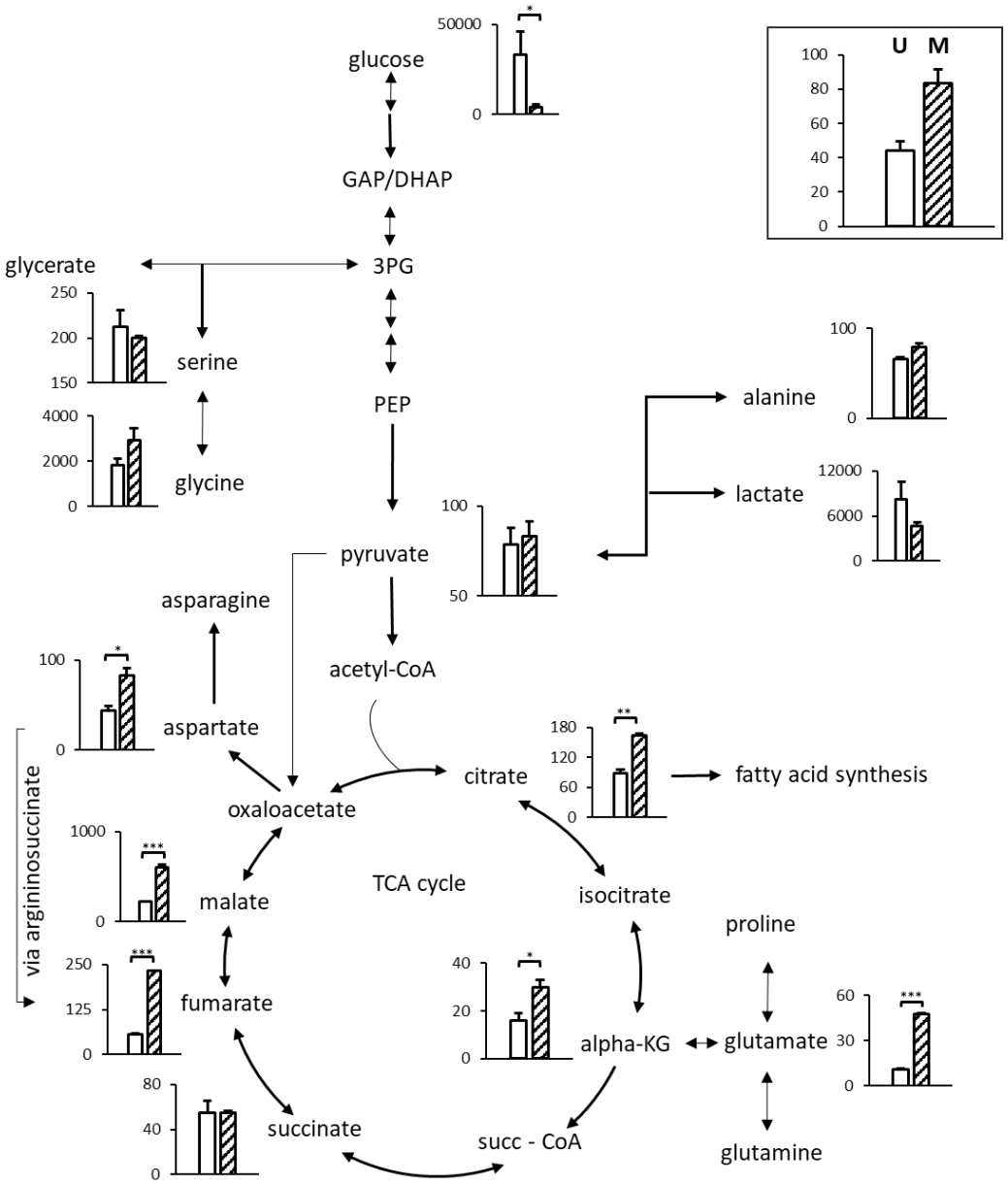

Figure 3.7. Differences in the pool sizes of metabolites of the central carbon metabolism after *S. aureus* NCTC 13626 infection of BALB/c Mouse Bone Marrow macrophages. Bone marrow macrophages were lysed after vancomycin-protection assays with NCTC 13626 (MOI 100; 6 hours) and metabolites were detected by GC-MS. Graphs show absolute levels of each metabolite in uninfected cells (white bars) and cells infected with NCTC 13626 strain (shaded bars). This figure shows means ± SE of two independent experiments performed in triplicates. Statistical differences were tested using Student´s t-test against uninfected cells. p-value ≤0.05 (*); ≤0.01 (**); ≤0.001 (***). Y-axis scale units are arbitrary units (x1,000).

3.2.3. Staphylococcal infection induces autophagy in HeLa cells

A number of the metabolic changes we observed in this study have previously been linked to autophagy, i.e. an increase in glutaminolysis and low levels of glucose and essential amino acids may activate the autophagic flux in mammalian cells (Russell, Yuan and Guan, 2014). Further, autophagy/xenophagy is a common host response against intracellular infection, where the autophagosomes are employed to engulf intracellular bacteria for later degradation. However, different studies have proved that *S. aureus* is able to survive and replicate within these autophagosomes before escaping to the cytosol of the cell (Fraunholz and Sinha, 2012).

Autophagy is commonly monitored by examining the conversion of the microtubule-associated protein light chain 3 (from LC3I to LC3II), since LC3II has been shown to directly correlate with the number of autophagosomes (Mizushima, 2009; Mizushima, Yoshimori and Levine, 2010). We therefore investigated autophagy status – the conversion of endogenous levels of LC3-I to LC3-II – by Western-blotting in our infection model (Fig. 3.8).

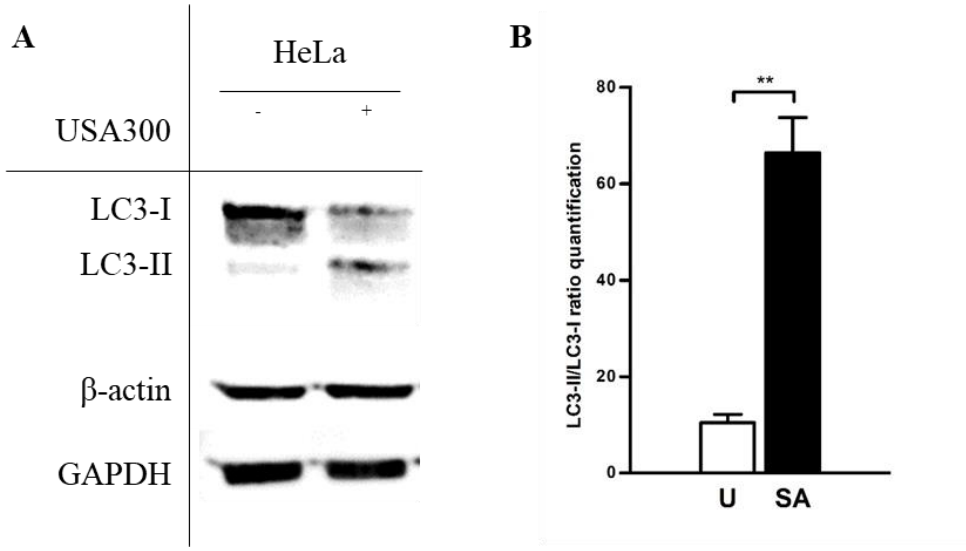

Figure 3.8. MRSA infection induces autophagy in HeLa cells. (A) HeLa cells were infected with USA300 strain (MOI 100; 6 hours) and protein lysates were analysed by Western-blot against LC3. The figure shows that MRSA infection promotes autophagy by activating the conversion of LC3-I to LC3-II. Both actin and GAPDH antibodies were employed as loading controls. The figure is a representative example of three independent experiments. (B) Quantification of the conversion of LC3-I to LC3-II in uninfected and infected-HeLa cells. Data are expressed as means ± SE of three different experiments and Student´s t-tests were performed to validate statistical significance across conditions. p-value ≤0.01 (**). U= uninfected cells; M= MRSA-infected cells.

Accordingly, the levels of conversion of LC3-I to LC3-II are markedly increased in MRSA-infected HeLa cells when compared to a non-infected control (Fig. 3.8). However, after careful analysis by confocal microscopy, we observed that cells with vacuoles staining positive for the auto-phagosomal marker mCherry-LC3 did not necessarily co-localize with bacteria (Fig. 3.9). Furthermore, 45% of intracellular bacteria were cytosolic at six hours post-infection, which may allow direct access to host metabolites present in the cytosol such as glucose (Fig. 3.10).

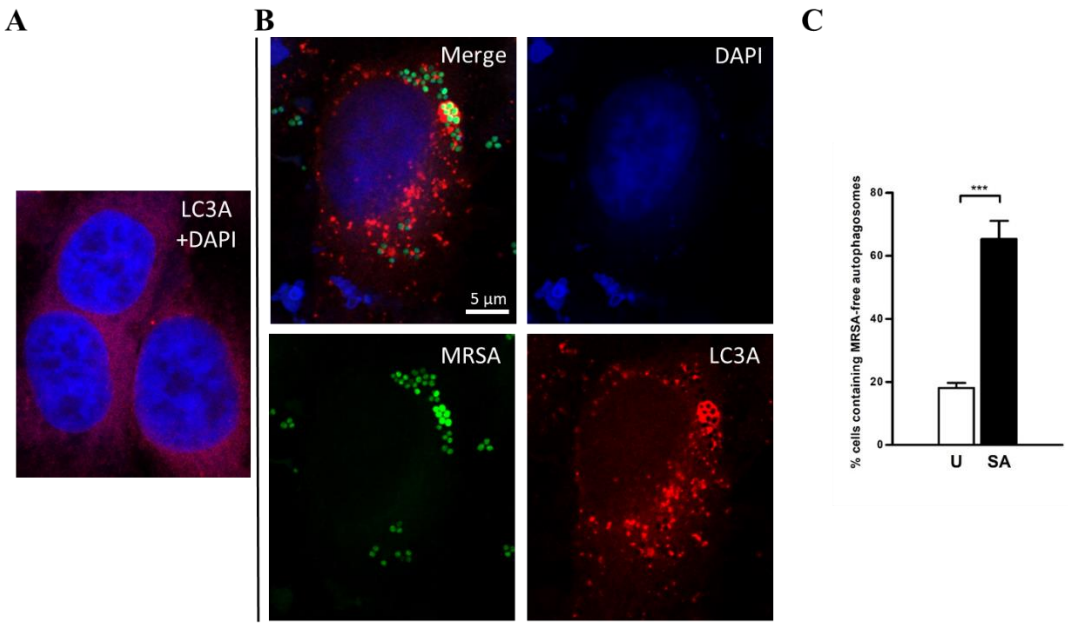

Figure 3.9. Colocalization assay of MRSA-GFP and mCherry-LC3A by confocal microscopy. (A) Uninfected HeLa cells expressing mCherry-LC3A. (B) HeLa cells expressing mCherry-LC3A infected with USA300-GFP strain (MOI 100; 6 hours); DAPI was employed for nucleus staining. The MRSA-free autophagosomes were detected around the whole cell. Bar indicates 5µm. (C) Quantification of percentage of cells containing MRSA-free autophagosomes in uninfected and infected-HeLa cells. Data are expressed as means ± SE of three different experiments and Student´s t-tests were performed to validate statistical significance across conditions. p-value ≤0.001 (***). U= uninfected cells; M= MRSA-infected cells.

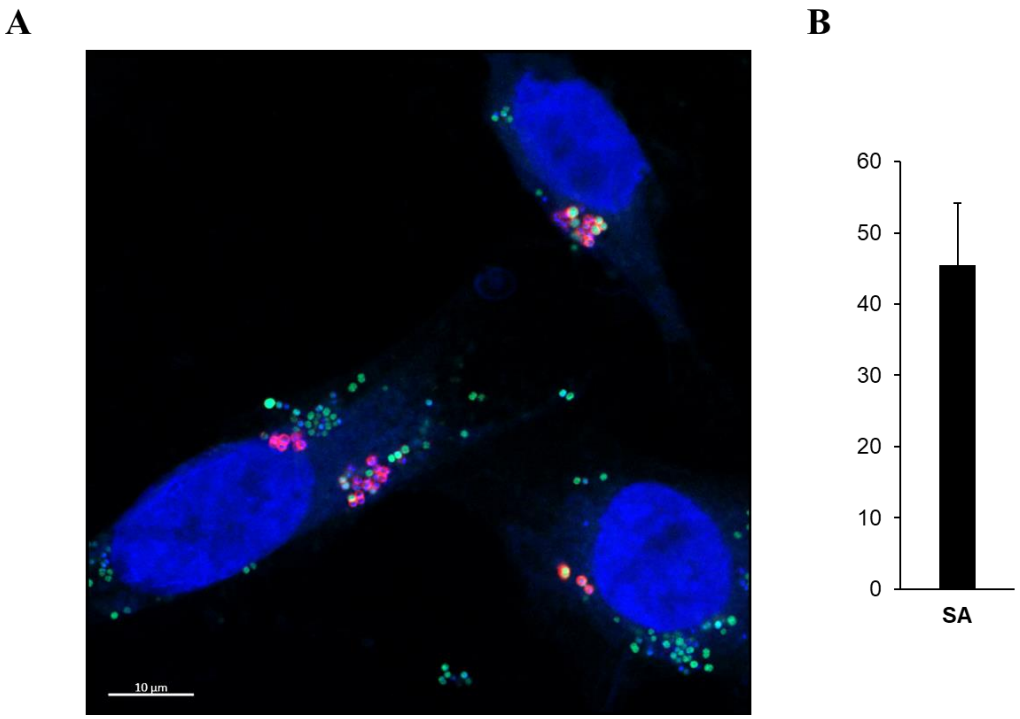

Figure 3.10. Co-localization assay of USA300-GFP and mCherry-CWT by confocal microscopy. (A) HeLa cells expressing the fluorescent escape marker mCherry-CWT were infected with USA300-GFP strain (MOI 100; 6h); DAPI was employed for nucleus staining. The recruitment of mCherry-CWT (red) to the bacterial cell wall of USA300-GFP (green) identifies cytosolic bacterial cells. (B) Quantification of percentage of USA300-GFP cells labelled with mCherry-CWT. Data are expressed as means ± standard errors of three different experiments. SA= *S. aureus*-infected cells.

3.2.4. AMPK and ERK1/2 pathways are activated in HeLa cells after MRSA infection.

The low levels of glucose, the activation of glutaminolysis and the drop of some amino acid levels in HeLa cells infected with USA300 point towards starvation and this may explain the induction of autophagy by the pathogen. As one of the main roles of autophagy is degradation of organelles for energy generation and nutrient scavenging (Rabinowitz and White, 2010), we have studied the phosphorylation state of AMPK and ERK during MRSA intracellular infection, which are sensors

of energy stress and amino acid deprivation in mammalian cells. Particularly, AMP-activated protein kinase (AMPK) is a key regulator in cellular energy homeostasis in eukaryotes and this pathway is activated when intracellular levels of ATP are lower (Carling *et al.*, 2011; Mihaylova and Shaw, 2011). Furthermore, a drop of amino acids in eukaryotic cells has been linked to a stimulation of autophagy by activating the Ras/Raf1/Erk1/2 pathway (Goldsmith, Levine and Debnath, 2014).

We investigated the role of AMPK and Erk1/2 in HeLa cells and we found that both pathways were activated in response to MRSA infection in HeLa cells. Basal levels of Erk1/2 and AMPK were increased in HeLa cells infected with MRSA; however, levels of the phosphorylated version of both proteins was even higher (Fig. 3.11A). Thus, ratios of phospho-AMPK/AMPK and phospho-Erk2/Erk2 were 4 and 1.7 times higher, respectively in MRSA-infected cells (Fig. 3.11B).

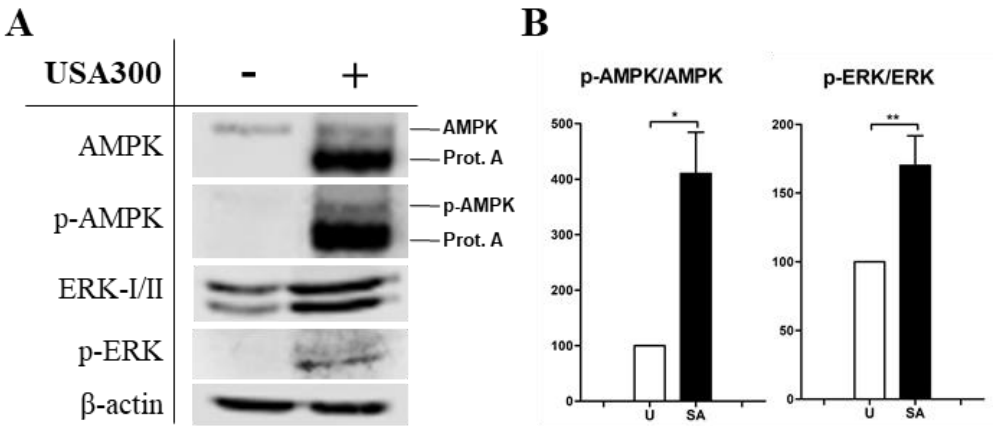

Figure 3.11. Activation of AMPK and ERK2 pathway was detected in MRSA-infected cells. HeLa cells were infected with USA300 (MOI 100; 6 hours). (A) Protein lysates were analysed by Western-blot against AMPKα (AMPK), phosphor-AMPKα (p-AMPK), p44/42 MAPK (ERK1/2) and phospho-p42/44 MAPK (p-ERK1/2). β-actin antibody was employed as loading control. (B) Quantification of the ratio phospho-AMPK/AMPK and phospho-Erk2/Erk2. Data were normalized to β-actin levels for each condition. Data are expressed as means ± SE of three different experiments and Student´s t-tests were performed to validate statistical significance across conditions. p-value ≤0.05 (*), ≤0.01 (**). U= uninfected cells. M=MRSA-infected cells.

To further validate these results, we tested the effect of dorsomorphin – an AMPK inhibitor (Zhou *et al.*, 2001) – on MRSA infection. We first confirmed that dorsomorphin has no direct effect on USA300 growth (Fig. 3.12).

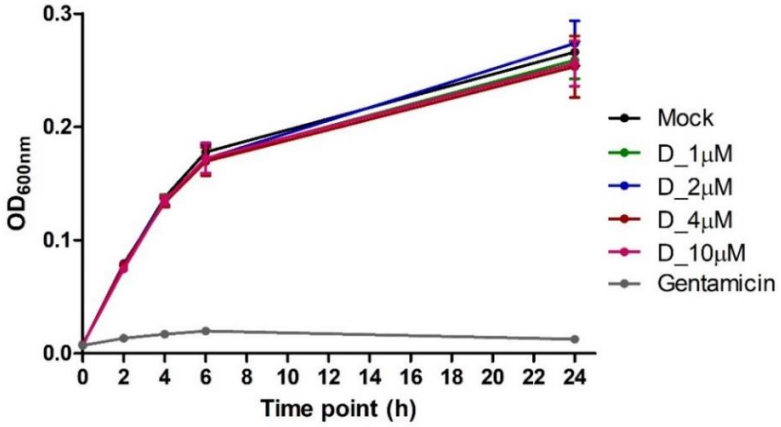

Figure 3.12. Dorsomorphin does not have a direct effect on *S. aureus* growth. MRSA USA300 was grown in 96-wells plates in the presence of DMSO (Mock), Dorsomorphin at different concentrations ranging from 1-10 μM, or gentamicin. Absorbance (OD_{600nm}) was measured at 2, 4, 6 and 24 hours to produce the growth curves. Data are expressed as means ± SE of three different experiments performed in triplicates. D, Dorsomorphin.

We then tested the effect of dorsomorphin on the cell viability of USA300-infected cells. High concentrations of dorsomorphin did not restore host cellular viability during infection, indicating this drug can be cytotoxic at high doses to mammalian cells (Fig. 3.13A). However, in the presence of 2 μM dorsomorphin, host cell viability was increased in comparison to non-treated control, whereas intracellular MRSA survival was inhibited after 6 hours of infection in HeLa cells (Fig. 3.13A-B). Furthermore, the levels of conversion of LC3-I to LC3-II were also significantly reduced in dorsomorphin-treated cells compared to non-treated cells after MRSA infection (Fig. 3.13C-D).

Additionally, we investigated the effect of AMPK inhibition on HUVEC, an endothelial cell line in which *S. aureus* has also shown to survive intracellularly (Strobel *et al.*, 2016). In this cell line, host cell viability was increased in the

presence of dorsomorphin, in a similar manner as in HeLa cells (Fig. 3.13E). On the other hand, the effect of AMPK inhibition on MRSA intracellular survival was more pronounced in HUVEC than in HeLa cells (Fig. 3.13F).

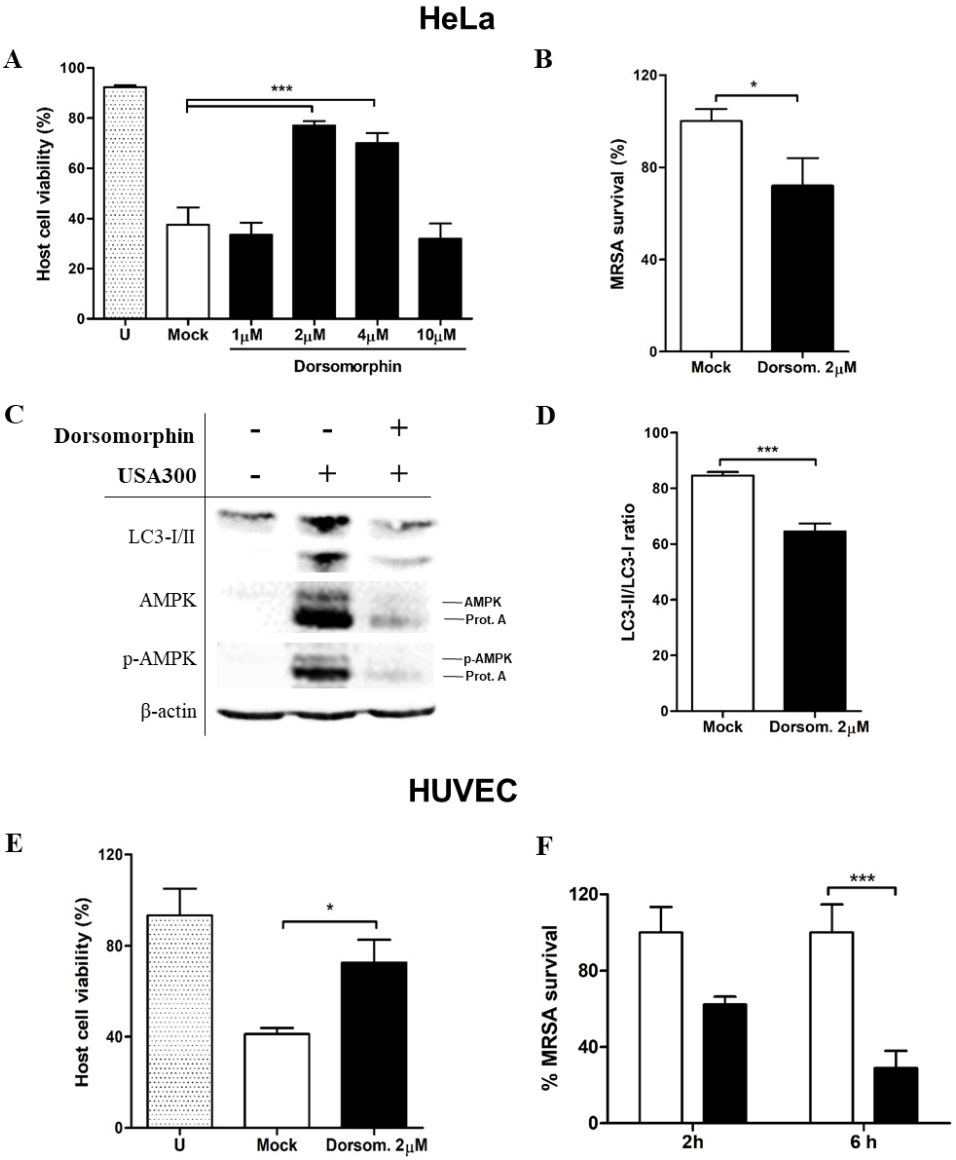

Figure 3.13. AMPK inhibition increases cell viability while reduces intracellular MRSA survival and autophagy levels. HeLa cells and HUVEC were infected with USA300 (MOI 100; 6 hours) in the presence or absence of dorsomorphin. (A & E) Host cell viability after USA300 infection was quantified by flow cytometry in HeLa cells and HUVEC, respectively. (B & F) Intracellular MRSA survival was measured by CFU counting in agar plates in HeLa cells and HUVEC, respectively. (C) Protein lysates were analysed by Western-blot against LC3I/II, AMPKα (AMPK), phosphor-AMPKα (p-AMPK). β-actin antibody was employed as loading control. (D) Quantification of the conversion of LC3-I to LC3-II. Data are expressed as means ± SE of three different experiments and Student´s t-tests were performed to validate statistical significance across conditions. p-value ≤0.05 (*), ≤0.01 (**), (***) ≤0.001.

3.2.5. Analysis of lipid-bound fatty acids

Fatty acids could also act as powerful signalling molecules and be involved in different metabolic processes during intracellular MRSA infection (Quehenberger, Armando and Dennis, 2011). Thus, we determined the levels of esterified fatty acids in MRSA-infected cells.

We quantified ten fatty acids in our samples. Seven of them showed no differences among treatments, such as stearic acid. However, the levels of three long-chain saturated fatty acids were markedly increased during intracellular MRSA infection: palmitic, docosanoic and eicosanoic acid (Fig. 3.14). Absolute levels of eicosanoic acid increased in cells exposed to heat-killed USA300 bacteria but were even higher in cells infected with alive USA300 (Fig. 3.14).

On the other hand, the carbon labelling distribution varies across the three experimental conditions, especially for docosanoic and eicosanoic acids. In MRSA-infected cells, both eicosanoic and docosanoic acids showed a similar labelling distribution, displaying an increase in glucose-derived label (Fig. 3.12). These two free fatty acids are elongation products obtained from intermediates of the glycolysis pathway, hence the rise in glucose-label is in accordance what we would expect. However, the percentage increase in abundance outstrips the percentage increase in glucose label influx, suggesting other – yet unknown – contributing factors.

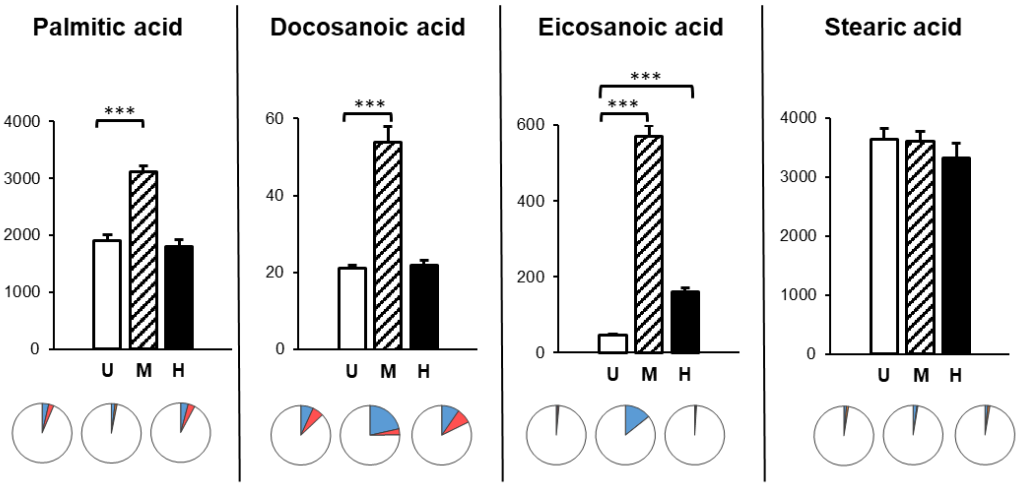

Figure 3.14. Differences in lipids metabolism in HeLa cells after USA300 infection. HeLa cells were infected with USA300 and exposed to heat-killed USA300 strains (MOI 100; 6 hours) and fatty acids levels and their labelling distribution were detected by GC-MS. Bar graphs show absolute levels of each fatty acid in uninfected cells (white bars), cells infected with USA300 strain (shredded bars) and cells exposed to heat-killed USA300 strain (black bars). Pie charts display the labelling pattern of each fatty acid in uninfected cells (left pie-chart), cells infected with USA300 strain (middle pie-chart) and cells exposed to heat-killed USA300 strain (right pie-chart). Within pie-charts, blue slides represent carbon coming from glucose-labelled, whereas red slides are carbon coming from glutamine-labelled and white slides show carbon from other sources. Data are expressed as means ± SE of two independent experiments performed in triplicates and Student´s t-tests were performed to validate statistical significance across conditions. p-value ≤0.05 (*); ≤0.01 (**); ≤0.001 (***). Y-axis scale units are arbitrary units (x1,000).

We then evaluated the impact of these three fatty acids on MRSA infection by measuring the host cell viability during infection assays and the bacterial growth in medium supplemented with 30 µM of palmitic, docosanoic or eicosanoic acid (Fig. 3.15). We used stearic acid as an additional control, since the levels of this fatty acid were not increased in MRSA-infected cells (Fig. 3.14).

When compared to the negative control, the host cell viability was significantly increased during infection assays when DMEM was supplemented with either palmitic, docosanoic or eicosanoic acid, but not with stearic acid (Fig. 3.15A). In addition, bacterial growth at 6 and 8 hours was halted in the presence of both docosanoic and palmitic acids, and at 24 hours bacterial growth was attenuated by

all the three cytoprotective fatty acids (Fig. 3.15B), but not by the presence of stearic acid in the medium. These results suggest that the increase of host cell viability after MRSA infection is due to the directly reduction of MRSA growth in the presence of these three fatty acids.

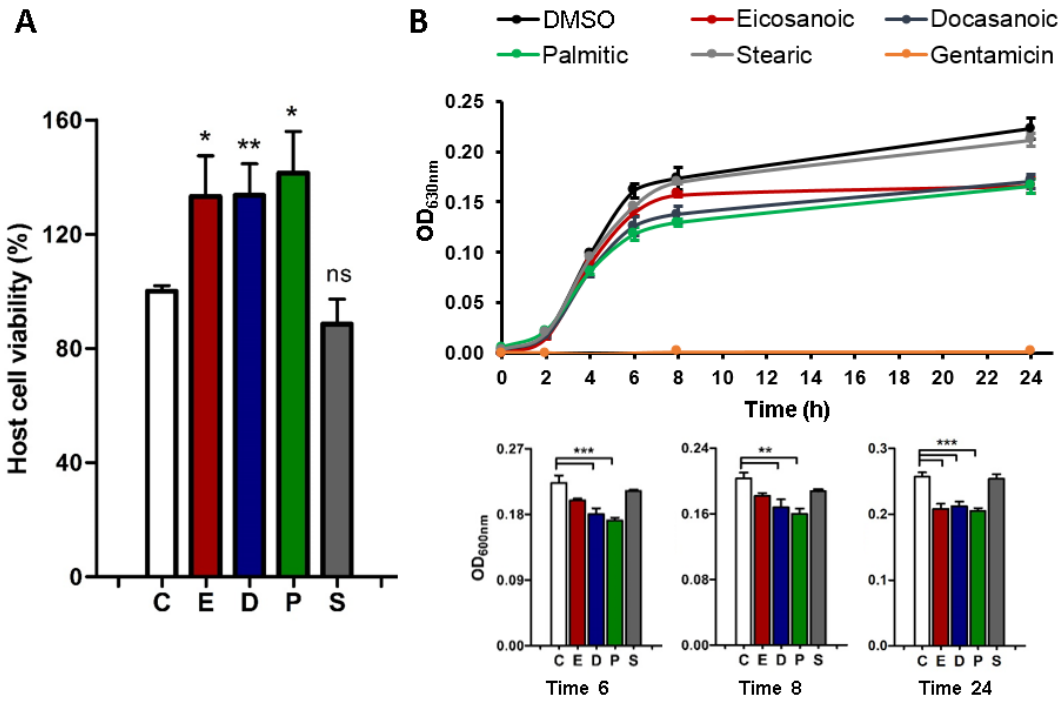

Figure 3.15. Three fatty acids increase host cell viability after USA300 infection by decreasing growth. HeLa cells were infected with USA300 (MOI 100; 6 hours) in the presence of DMSO (C), eicosanoic acid (E), docosanoic acid (D), palmitic acid (P) and stearic acid (S), and host cell viability as well as bacterial growth were tested. (A) Quantification of host cell viability after USA300 infection, measured by flow cytometry using double annexin V-FITC and PI staining. (B) Bacterial growth curves in NB (up) and the growth levels at 6, 8 and 24 hours (down). Data are expressed as means ± SE of three independent experiments performed in duplicates. Statistical significance was analysed by one-way ANOVA and post hoc Tukey's multiple comparison tests. p-value ≤0.05 (*); ≤0.01 (**); ≤0.001 (***).

3.3. DISCUSSION

3.3.1. MRSA intracellular infection leads to extensive metabolic re-routing leading to the host exhibiting signs of metabolic starvation

In this study, we were interested in the metabolic changes brought about by intracellular *S. aureus* infection of mammalian cells. I performed NMR and MS-based profiling and found that intermediates of lower glycolysis – such as phosphoenolpyruvate (PEP) and 3-phosphoglycerate (3-PG) – were highly increased in HeLa cells infected with the USA300 strain of *S. aureus*. Although the absolute levels of PEP and 3-PG were raised in HeLa cells after infection with the USA300 strain, the levels of lactate were relatively unaffected in both intracellular and extracellular compartments. Interestingly, the levels of intracellular pyruvate were (slightly, compared to PEP) increased in HeLa cells infected with the USA300 strain and the secretion of this metabolite to the medium was also elevated in infected cells. This is suggestive of a down-stream block explaining the accumulation of PEP and 3-PG. While the nature of this block is unknown, potential targets include a (partial) inhibition of pyruvate kinase, the mitochondrial pyruvate carrier and/or the pyruvate dehydrogenase complex as well as regulatory interference of pyruvate dehydrogenase kinases.

In stark contrast, MRSA-infected cells excreted vastly increased quantities of acetate and formate. Acetate excretion is not uncommon in HeLa cells infection models as a product of bacterial activity (Kentner *et al.*, 2014) and our findings suggest elevated flux through bacterial pyruvate-formate lyase (Pfl). Pfl generate formate and acetyl-CoA, which has been shown to be converted to acetate via phosphate acetyltransferase (Pta) and acetate kinase (AckA) for ATP generation

(Halsey *et al.*, 2017) in some conditions. Furthermore, the increase of excretion (Fig. 3.1) as well as labelling pattern in USA300-infected cells (Fig. 3.3) for both formate and acetate is similar to each other and to that of pyruvate, suggesting a common source. These results suggest that despite higher glucose uptake in infected cells, bacterial metabolism might subvert some of the carbon intake, possibly contributing to a starvation response. While Pfl activity has mainly been activated under anaerobic conditions and in biofilms (Leibig *et al.*, 2011), intracellular Pfl activity might be a hallmark of intracellular MRSA/*S. aureus* proliferation but further investigation is needed to confirm this hypothesis.

Among all the metabolic changes generally seen in a host cells after an intracellular infection, alterations of the TCA cycle are relatively common (Eisenreich *et al.*, 2013, 2015). The TCA cycle represents an important source of carbons and chemical energy for intracellular pathogens (Abu Kwaik and Bumann, 2015), therefore it is likely that intracellular pathogens exploit this pathway for their intracellular survival and proliferation. In this study, the TCA cycle was greatly affected by MRSA infection, regardless the strain or cell line employed. While glutamine was the main source of carbon for most of the intermediates, there was a clear rise in the glucose-derived label in fumarate and aspartate of infected cells. The reason for this difference in label distribution between fumarate/aspartate and succinate is not fully clear but could arise via several pathways. As the increase in glucose-derived label was mainly due to an increase in the M+3 ion (Fig. 3.4), this suggests an anaplerotic flux from either pyruvate or PEP. One possibility is an increased flux through pyruvate carboxylase forming oxaloacetate, potentially as a consequence of reduced flux through succinate dehydrogenase (Sdh), as previously observed in a study looking at the metabolic effects of loss-of-function *sdh* mutants

(Lussey-Lepoutre et al. 2015). Moreover, the same study also found amino acid pool size alterations similar to those seen in the current study (Lussey-Lepoutre et al. 2015). Given the high levels of PEP, it is also possible that the reaction of PEP carboxykinase might be reversed (it usually catalyses the decarboxylation of oxaloacetate to PEP during gluconeogenesis) (Yang, Kalhan and Hanson, 2009). On its own, however, this would not explain the difference between fumarate and malate labelling patterns. In addition, the increase in glutaminolysis that we have detected will increase the availability of ammonium, which can be used to convert oxaloacetate to aspartate, and this in turn is converted to fumarate in the urea cycle. Activation of the urea cycle is imminently plausible due to increased amounts of glutamate, an activator of N-acetylglutamate synthase, the product of which, N-acetylglutamate, is a key positive regulator of urea cycle activity (Caldovic and Tuchman, 2003).

3.3.2. MRSA-induced starvation initiates an autophagy response that is exploited by the bacteria

Several host metabolic changes observed in MRSA-infected cells pointed towards starvation and this condition commonly leads to autophagy. Since autophagy has already been described to benefit some intracellular pathogens, we explored whether host-autophagy was implicated in intracellular MRSA infection.

Among all its physiological functions, the role of autophagy as a defence against intracellular pathogens has been widely described (Levine, 2005; Mizushima, 2009; Deretic, Saitoh and Akira, 2013; Wileman, 2013). However, intracellular pathogens have developed refined mechanisms to subvert autophagy and successfully survive

within mammalian host cells (Colombo, 2005; Kimmey and Stallings, 2016; Casanova, 2017). For instance, *S.* Typhimurium and *M. tuberculosis* inhibit the autophagy initiation signalling (Shin *et al.*, 2010; Tattoli *et al.*, 2012), whereas *S. flexneri* escapes from autophagy recognition by recruiting Toca-1 via IcsB to inhibit recognition by autophagy machinery (Ogawa, 2005; Krokowski and Mostowy, 2016). In contrast, *S. aureus* is able to replicate in the autophagosomes and to eventually escape to the host cytosol (Schnaith *et al.*, 2007; Neumann *et al.*, 2016), although the molecular mechanisms behind this are not yet fully understood. However, our analysis of LC3 and MRSA co-localization by confocal microscopy revealed that a significant number of autophagosomes do not contain bacteria in MRSA-infected cells, suggesting that these autophagosomes may have been formed for an alternative purpose.

Autophagy-inhibition with wortmannin impairs the intracellular proliferation of *S. aureus*, whereas the induction of autophagy by rapamycin results in an increased number of intracellular bacteria (Schnaith *et al.*, 2007). On the other hand, autophagy confers cellular protection to the α-toxin produced by *S. aureus* (Maurer *et al.*, 2015). However, the α-toxin is not sufficient to permeabilize phagosomes in HeLa cells, probably reflecting expression differences in the receptor of α-toxin monomers: the ADAM10 metalloprotease (Lâm *et al.*, 2010; Fraunholz and Sinha, 2012; Inoshima *et al.*, 2012). Therefore, we have hypothesized here that MRSA-free autophagosomes could be produced in response to a different autophagy-inducing stimulus other than leaky phagosomes containing bacterial cells or the α-toxin produced by the pathogen.

The role of autophagy as a host-defence pathway against intracellular pathogens has been already questioned, since the replication of some bacteria was impaired in

autophagy-deficient cells and the treatment with autophagy-activators recovered bacterial replication (Mostowy and Cossart, 2012). Considering that autophagy could increase the nutrients access, intracellular pathogens may have evolved some mechanisms to manipulate host-autophagosomes and to exploit the products of autophagic degradation for bacterial survival and proliferation (Steele, Brunton and Kawula, 2015; Escoll, Rolando and Buchrieser, 2016). Intracellular MRSA causes multiple changes in cellular metabolism during infection and many of the changes we observed point towards a situation of starvation, probably induced by the pathogen. This state of induced-starvation might also explain the presence of numerous MRSA-free autophagosomes.

In response to nutrient deprivation, autophagy is induced in mammalian cells (Rabinowitz and White, 2010; Goldsmith, Levine and Debnath, 2014). This process is mainly regulated by mammalian target of rapamycin (mTOR), which acts as sensor of the host metabolic state (Kroemer, Mariño and Levine, 2010). In a wide range of mammalian cell lines, low levels of glucose enhance the autophagic machinery (Goldsmith, Levine and Debnath, 2014), whose regulation is usually dependent on AMPK signalling (Williams *et al.*, 2009). During glucose deprivation, the proautophagy Beclin 1/VPS34 complex is activated via AMPK-dependent Beclin 1 phosphorylation (Kim *et al.*, 2013). Moreover, a drop in the amino acids levels also induce autophagy in mammalian cells via Ras/Raf-1/ERK1-2 signalling pathway (He and Klionsky, 2009; Goldsmith, Levine and Debnath, 2014). Additionally, in response to low levels of glucose, glutaminolysis is activated to sustain the TCA cycle and ATP production (Goldsmith, Levine and Debnath, 2014) and as a consequence of the ammonia produced by the glutaminolysis pathway, the autophagic flux is increased (Eng *et al.*, 2010; Cheong,

Lindsten and Thompson, 2012). In HeLa cells infected with the USA300 strain, all these starvation signals were present: reduction in the levels of glucose, drop of several amino acids and activation of glutaminolysis pathway. Interestingly, these changes were also observed in both HeLa cells and BALB/c mouse bone marrow macrophages infected with MRSA NCTC 13626 strain, suggesting that the results previously mentioned are not just strain and host dependant. In addition, we also observed an activation of both AMPK and ERK signalling pathway in HeLa cells after MRSA infection. In accordance with these findings, a recent study has shown that the levels of ATP in human alveolar epithelial cells were significantly decreased between two and six hours of *S. aureus* infection (Gierok *et al.*, 2016), further pointing to a starvation-induced response. Crucially, once we inhibited the host-AMPK pathway using dorsomorphin, intracellular MRSA survival was significantly hampered, and autophagy levels were also reduced, supporting our hypothesis that the activation of autophagy is caused by the state of starvation induced by the intracellular pathogen, which benefits the intracellular survival of MRSA. Future studies need to be carried out to identify additional components of this regulatory, but our findings suggest a novel host-directed anti-staphylococcal strategy based on the inhibition of AMPK-mediated autophagy.

3.3.3. Increase in the levels of three saturated fatty acids in HeLa cells in response to MRSA infection

Lipid droplets (LDs) are cellular organelles where triglycerides (TGs) and steryl esters (STEs) are stored and surrounded by a phospholipids and protein monolayer. LDs are considered the main lipid reservoir in eukaryotic cells (Martin and Parton,

2006). Recent studies have pointed the link between LDs and autophagy since this cellular process is able to degrade triglycerides (TGs) and cholesterol from LDs (Singh *et al.*, 2009; Cingolani and Czaja, 2016). Also, many LD-associated proteins are related to membrane trafficking and particularly in HeLa cells, the requirement of LDs and their interactions with early autophagosomes has been shown (Dupont *et al.*, 2014). Furthermore, in starved-cells there is an increase in the number of LDs over time (Rambold, Cohen and Lippincott-Schwartz, 2015). The observed accumulation of LDs in starved cells and its complex link with the autophagosomes biogenesis may explain the increase in the absolute levels of some fatty acids in cells infected with MRSA. These results are in line with the starvation-induced autophagy, probably in response to the intracellular MRSA infection. Additionally, some studies have shown that palmitic acid stimulates autophagy, mediated by JNK1 and PKC activity and independent from mTOR pathway (Tan *et al.*, 2012; Goldsmith, Levine and Debnath, 2014).

The absolute levels of eicosanoic acid were also significantly increased in HeLa cells exposed to heat-killed bacteria, suggesting that part of this increase is due to a host-immune response. Considering that eicosanoids have been related to inflammation and are considered a main component of the host innate immune responses (Dennis and Norris, 2015), the rise of eicosanoic acid in the host cell in response to heat-killed bacteria exposure is not surprising. In relation to the labelling distribution, the increase in glucose-derived label of both eicosanoic and docosanoic acids – whose labelling pattern is very similar to each other – agrees with the fact that elongation of fatty acids is driven from Acetyl-CoA (AcCoA) and thus, glycolysis (Pietrocola *et al.*, 2015).

The cytoprotective role of palmitic, docosanoic and eicosanoic acids that we have observed during MRSA infection is directly related to the bacterial killing as there is a significant reduction of MRSA when cultured in the presence of these three saturated fatty acids. The bactericidal role of fatty acids against Gram positive and Gram negative bacteria has been widely studied (Desbois and Smith, 2010). Although some studies have shown bactericidal activity of unsaturated acids on *S. aureus* growth (Desbois and Smith, 2010; Cartron *et al.*, 2014) not many have investigated the role of saturated fatty acids and their effect on *S. aureus* survival.

The specific answer of how fatty acids mediate their antibacterial activity remains still unclear. The key membrane-associated process altered by the presence of fatty acids is the production of energy through the disruption of the electron transport chain and oxidative phosphorylation. However, fatty acids could also inhibit bacterial growth by promoting cell lysis, inhibiting enzyme activity or impairing nutrient acquisition from the host cell (Desbois and Smith, 2010). Our study shows that the presence of palmitic, docosanoic and eicosanoic significantly affect MRSA growth in vitro, reducing bacterial growth. Nevertheless, the precise mechanism underlying the antibacterial activity of these lipids is unknown and further experiments need to be carried out to unravel the mechanistic behind.

The combination of fatty acids along with existing therapies may increase their effect and could contribute to the reduction of antimicrobial resistance. The combination of free fatty acids and cholesteryl esters have been recently tested against *P.aeruginosa* and *S. epidermis* and results have shown a potential as novel antimicrobial agents (Cheung Lam *et al.*, 2016).

3.4. CONCLUSION

In summary, we observed that autophagy in host cells is activated in response to intracellular MRSA-infection. During intracellular MRSA proliferation, the levels of glucose and several amino acids become significantly low in the host cell (likely due to bacterial metabolic activity), whereas glutaminolysis is highly activated. These observations are indicative of a state of starvation induced by the pathogen, which would explain the production of MRSA-free autophagosomes observed in infected host cells. This hypothesis is also supported by the activation of AMPK and ERK pathways, which act respectively as a key sensors of cellular energy homeostasis and amino acid deprivation.

In addition, the presence of this intracellular pathogen also alters the host-lipidome. We found that the levels eicosanoic, docosanoic and palmitic acids significantly increased in MRSA-infected cells. Interestingly, the presence of these three fatty acids seems to have a cytoprotective role in HeLa cells after MRSA infection, due to direct reduction of MRSA *in vitro*.

4. Host-directed kinase inhibitors act as novel therapies against intracellular *Staphylococcus aureus*

4.1. BACKGROUND

Current approaches to manage staphylococcal infections are focused on targeting specific processes, pathways or enzymes of the pathogen. The main advantage of this strategy is the minimal host toxicity. However, the major disadvantage of pathogen-directed therapies is the development of multidrug microbial resistance. This emerging and serious health issue requires the development of either effective vaccines or the identification of novel targets against multidrug resistant pathogens (Schwegmann and Brombacher, 2008).

As part of their host-defense evasion mechanism, intracellular pathogens subvert and exploit a wide range of host factors and pathways to support their intracellular survival (Finlay and Cossart, 1997). They are able to target multiple pathways to assure their intracellular proliferation (Bhavsar, Guttman and Finlay, 2007). In the case of *S. aureus*, it has been shown that host-integrins are required for the

internalization of *S. aureus* into non-phagocytic cells (McDonnell *et al.*, 2016). This interaction further leads to a cascade of signal transduction in which several host genes and pathways, such as FAK, Src, PI3-Akt, are involved (Agerer, 2005; Oviedo-Boyso *et al.*, 2011). Host actin and cytoskeleton rearrangement has also been identified to play an important role during staphylococcal infection. Intracellular *S. aureus* induced the formation of actin comet tails, by recruiting Neural Wiskott-Aldrich syndrome N-WASP protein, to facilitate bacterial movements during invasion to endothelial cells (Schröder *et al.*, 2006). Similarly, there are other host genes that have been described to be important at different points of intracellular staphylococcal infection, including bacterial invasion, intracellular persistence or host cell death induced by the pathogen (Fraunholz and Sinha, 2012; Horn *et al.*, 2017).

Therefore, the study of host-pathogen interactions may lead to the identification of novel pathogen-specific drug targets and/or host-directed therapeutics. Host-directed approaches could: (i) interfere with the host-pathways exploited by intracellular pathogen to survive within the host cell, (ii) enhance the immune response by stimulating host-defense responses against intracellular infection, (iii) target those pathways that cause hyper-inflammation and (iv) adjust host factors that lead to unstable responses at the site of infection (Kaufmann *et al.*, 2018). Specifically, host-directed strategies comprise a range of different therapeutic agents such as monoclonal antibodies, vitamins, cytokines, cellular therapy, recombinant proteins and repurposed drugs (Zumla *et al.*, 2016).

Repurposing commercially available drugs that target specific host-pathways hijacked by intracellular pathogens is a particularly interesting strategy. The main advantage of using repurposed drugs is that they show minimal toxicity to the host-

cell and they have already been approved for other clinical purposes, which would importantly reduce the necessary time to have these drugs in the market (Schwegmann and Brombacher, 2008; Czyz *et al.*, 2014).

There are currently several repurposed drugs that are in preclinical development to treat bacterial and viral infections. For instance, dasatinib – a tyrosine kinase inhibitor – inhibits the replication of Dengue virus via blockade of host proto-oncogene kinase FYN (de Wispelaere et al. 2013). Imatinib – and inhibitor of BCR-ABL tyrosine kinase – reduces bacterial load and pathology in murine lungs infected with *Mycobacterium tuberculosis* (Napier *et al.*, 2015). In addition, cholesterol inhibitors has shown potential activity against *S. aureus* infection (Liu *et al.*, 2008) since it is needed for internalization of the bacterium and the activation of virulence factors (Schwegmann and Brombacher, 2008).

Moreover, we recently discovered that host-autophagy is induced by MRSA through the activation of the AMPK pathway. Our study showed a significant reduction of intracellular bacterial load in both primary and established cell lines due to host-directed AMPK inhibition of dorsomorphin (Bravo-Santano *et al.*, 2018).

Based on these previous observations, here we screened for host-directed drugs that have already been approved for other clinical purposes, seeking to identify novel host-directed compounds to control the cell infection caused by *S. aureus*. Interestingly, we identified a host-kinase inhibitor – Ibrutinib – that hampered intracellular *S. aureus* survival and we investigated its mechanism of action by performing phosphoproteomics analyses.

4.2. RESULTS

4.2.1. Host-directed drugs screening against intracellular MRSA

We initially tested the effect on intracellular MRSA infection of 11 host-targeting drugs used to treat non-microbial diseases (Table 4.1). In particular, Amitriptyline-HCl, Cyproheptadine-HCl, Thioridazine-HCl and Trifluoperazine-2HCl are used to treat high blood cholesterol levels or psychosis, and have been previously shown to clear other intracellular pathogens (Czyz *et al.*, 2014). Hence, we tested these four compounds, along with other host-directed drugs, to investigate whether they have a similar effect on MRSA infection.

We aimed to find host-targeted therapeutics that would impair MRSA cell infection without affecting the host cell viability and thus, we first tested whether the selected drugs would show any cytotoxicity at a concentration of either 10 or 33 μM as previously reported (Czyż *et al.*, 2014) (Fig 4.1). We did not observe any significant increase in cell death in the presence of any drug at those concentrations except for Thioridazine-HCl and Trifluoperazine-2HCl, for which the minimal cell toxicity was only reached when using a concentration of 0.25 μM (Fig 4.1).

Drug	Mechanism of action	Clinical purpose
Lenalidomide	Antiangiogenic and antineoplastic	Multiple Myeloma
Pomalidomide	Immunomodulatory and antineoplastic	Multiple Myeloma
Bortezomib	Proteasome (26S) inhibitor	Multiple Myeloma
Ibrutinib	Bruton's Tyrosine Kinase inhibitor	Chronic Lymphocytic Leukaemia
Blebbistatin	Myosin inhibitor	Control of muscle contraction
Amitriptyline-HCl	G-protein coupled receptor	Depression
Cyproheptadine-HCl	G-protein coupled receptor and Ca2+ signals	Allergies
Niacin	Inhibition of TG synthesis	High blood cholesterol
Simvastatin	Induction of hepatic LDL receptors	High blood cholesterol
Thioridazine-HCl	Reduction of serotonin activity	Psychosis and Schizophrenia
Trifluoperazine-2HCl	Adrenergic receptors	Psychosis

Table 4.1.Pilot screen for host-directed drugs tested on *S. aureus* infection in HeLa cells.

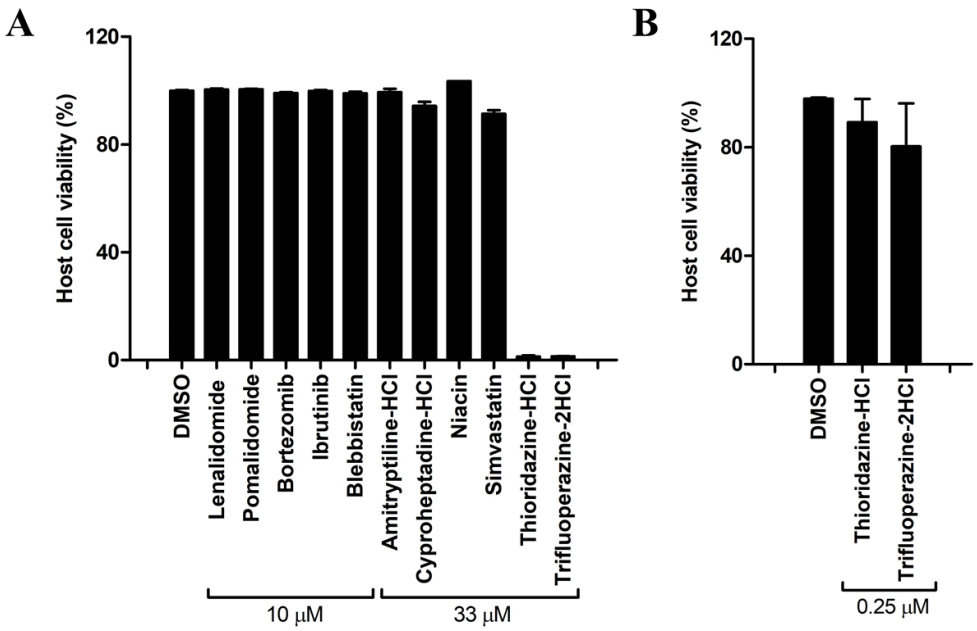

Figure 4.1. Effect of several host-directed drugs in HeLa cells viability. (A) HeLa cells viability was measured by flow cytometry in the presence of each drug after 6 hours of exposure, employing a double staining of Annexin V-FITC and Propidium iodide. (B) The highest viability of HeLa cells in the presence of Thioridazine-HCl and Trifluoperazine-2HCl was only reached at a concentration of 0.25 μM. Data are expressed as means ± SE of three independent experiments performed in duplicates.

In order to identify host-directed drugs against intracellular *S. aureus*, we hypothesized that if the drug hampers intracellular MRSA without causing any cytotoxic effect, host cell viability should be higher in comparison to untreated and MRSA-infected cells. Therefore, we measured the host cell viability after six hours of MRSA infection in the presence of DMSO (control) or specific drugs at the maximum concentration within the therapeutic window (Fig 4.2). Interestingly, we found that in the presence of Ibrutinib, cell viability was significantly increased (Fig. 4.2).

In contrast, we did not observe any significant results for the other 10 compounds we tested. In fact, cell viability under Trifluoperazine-2HCl treatment, which had a positive effect against *L. pneumophila*, *Brucella abortus*, and *Rickettsia conorii*

(Czyz *et al.*, 2014), was negatively affected by this treatment during *S. aureus* infection (Fig 4.2).

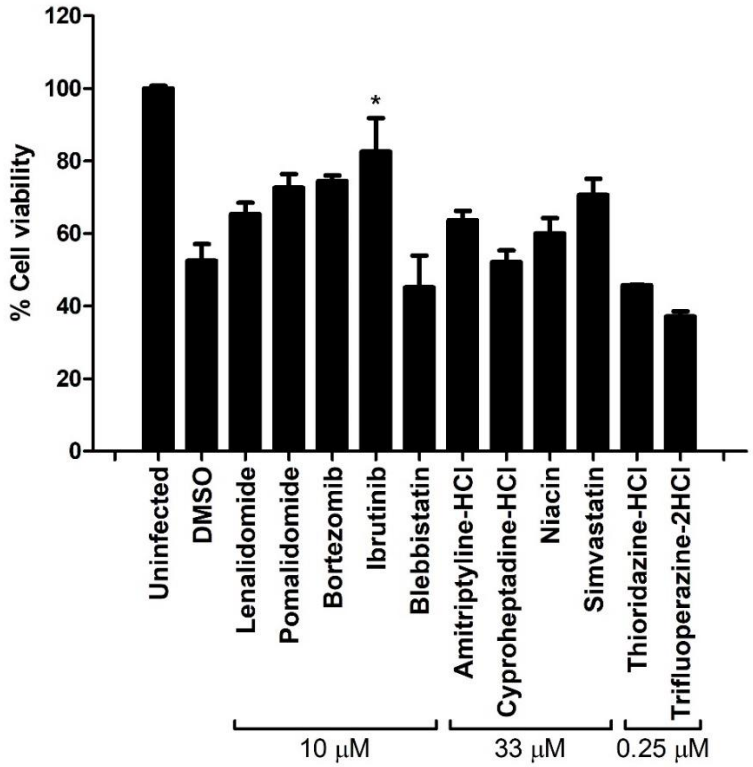

Figure 4.2. Host cell viability after *S. aureus* infection in the presence of different host-directed drugs. HeLa cells were infected with *S. aureus* USA300 (MOI 100; 6 hours) in the presence of different drugs. Host cell viability was measured by flow cytometry employing a double staining of Annexin V-FITC and Propidium iodide. Percentage of cell viability was normalized to untreated *S. aureus*-infected cells. Data are represented as means ± SE of two independent experiments performed in duplicates. One-way ANOVA and multiple comparison Tukey´s tests were performed to validate statistical significance across conditions. p-value ≤0.05 (*).

4.2.2. Developing a high-throughput screening for host-targeted therapeutics against intracellular *S. aureus*

To systematically screen a large number of drugs for their activity against staphylococcal invasion, we developed a fluorescence-based high-throughput assay (Fig. 4.3). Briefly, the assay uses flow cytometry to quantify viable host cells expressing the red fluorescent protein mCherry while also counting infected cells

with bacteria constitutively expressing the green fluorescent protein (GFP; Fig. 4.3A). After establishing the scalability of the approach (Fig. 4.3B), we used a length of infection of 6 hours with a multiplicity of infection (MOI) of 100 to screen the effect of 133 host-targeting FDA-approved drugs (Approved Oncology Drugs Set VIII – National Cancer Institute, US) on host and bacterial cell viability.

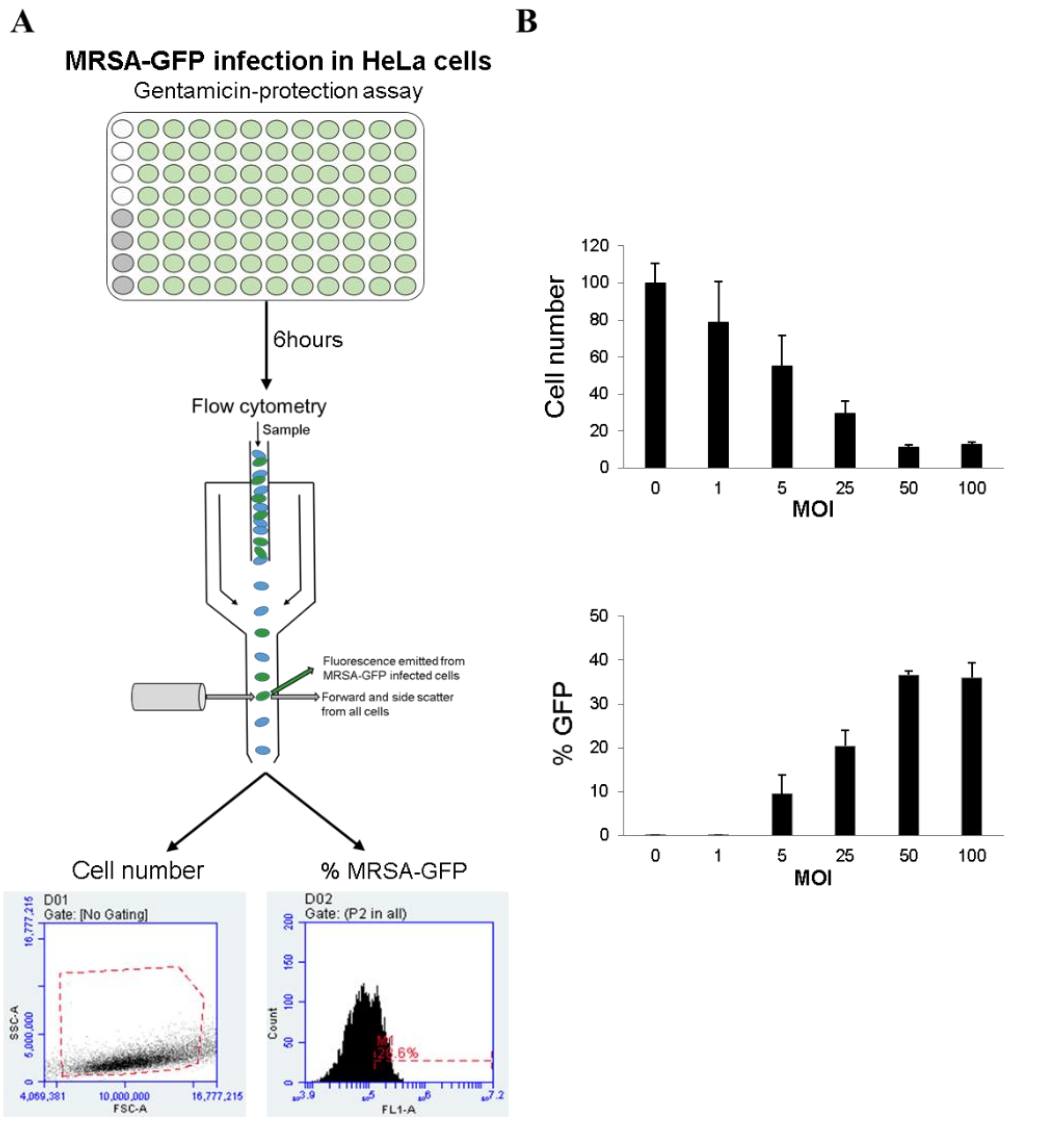

Figure 4.3. Host-directed drug-screening approach and standardization. (A) Experimental design of the drug screening approach. Untreated samples were always included as internal controls in each plate: four uninfected (white circles) and four MRSA-infected (grey circles). Each drug was added in a different well (green circles). After 6 hours of infection, samples were analyzed in the flow cytometer where cell number and percentage of cells containing *S. aureus*-GFP was quantified for every sample. (B) This approach was first standardized by using different MOIs. Data are expressed as mean ± SE of three independent experiments.

The effect of 10 μM of each drug is summarized in figure 4.4 and table 4.2. In order to identify the most interesting candidates, we established a restricted cut-off according to the following criteria: (a) 90% reduction of intracellular MRSA compared to the untreated host cells and (b) host cell viability of at least 80% compared to uninfected host cells. Following these two premises, we found three drugs within this threshold: Ibrutinib, Dasatinib and Crizotinib (Fig. 4.4B).

Of these three, Ibrutinib induced the clearest inhibitory effect on MRSA survival and the lowest variability across experimental replicates. Taken together, our data suggested that the host molecular pathway inhibited by Ibrutinib may play an important role in intracellular *S. aureus* infection and thus, we selected this drug for further validation and downstream experiments.

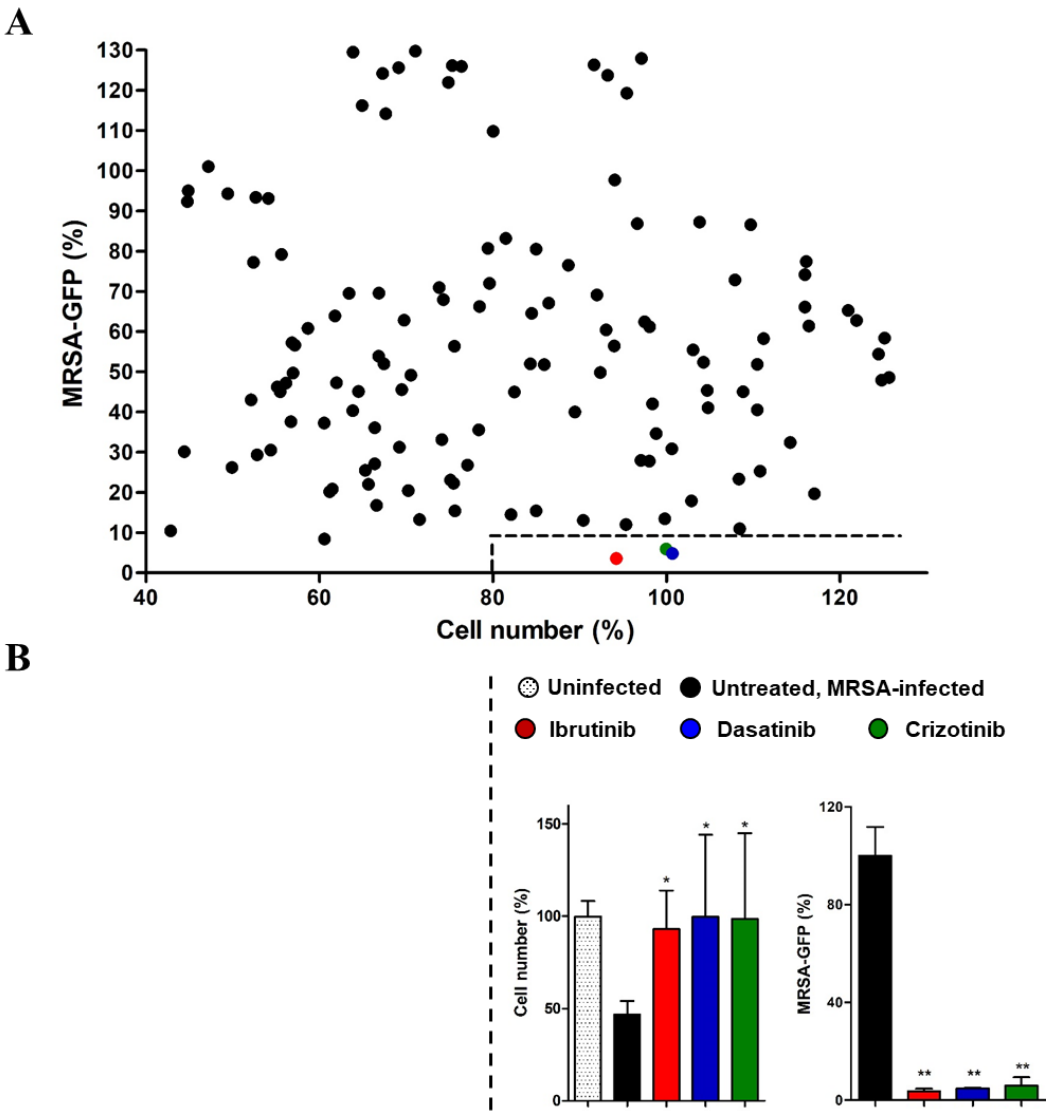

Figure 4.4. Host-directed drugs screening in HeLa cells after *S. aureus* infection. HeLa cells were infected with *S. aureus* USA300-GFP (MOI 100; 6 hours) in the presence of different drugs. (A) Host cell viability and percentage of HeLa cells containing USA300-GFP was measured by flow cytometry and normalized to uninfected cells and untreated *S. aureus*-infected cells, respectively. Each dot represents a different treatment. The dashed line in the right bottom corner depicts the established cut-off. (B) Percentage of cell viability and *S. aureus*-GFP for the three drugs within the cut-off values. Means ± standard errors (SE) of three different experiments are shown. p-value ≤0.05 (*); ≤0.01 (**).

Drug	Mean	SE	p-value	Mean	SE	p-value
Ibrutinib	93.12	17.17	0.0301	3.58	0.87	0.0073
Dasatinib	99.5	44.5	0.0405	4.84	0.16	0.0078
Crizotinib	98.94	46.62	0.0497	5.91	2.77	0.0083
Mitoxantrone	59.57	25.75	0.6609	8.42	0.72	0.0095
Ceritinib	41.84	3.55	0.2399	10.43	2.51	0.0000
Mitomycin	138.13	7.25	0.0002	10.94	1.34	0.0109
Capecitabine	107.42	29.04	0.0177	10.94	1.34	0.0109
Panobinostat	94.32	37.49	0.0837	12.01	1.59	0.0116
Afatinib	89.41	39.84	0.1310	13.08	4.51	0.0125
Gefitinib	70.54	10.14	0.2308	13.27	0.56	0.0124
Imatinib	98.78	17.61	0.0175	13.46	3.39	0.0127
Clofarabine	81.07	4.90	0.0695	14.52	0.47	0.0133
Mitotane	83.97	7.29	0.0528	15.40	6.41	0.0144
Teniposide	74.61	25.38	0.3498	15.41	6.62	0.0000
Nelarabine	65.57	12.88	0.3077	16.81	7.05	0.0045
Raloxifene	101.86	19.81	0.0150	17.92	4.36	0.0163
Plicamycin	116.02	11.61	0.0023	19.66	5.02	0.0002
Dexrazoxane	60.17	16.65	0.5481	20.20	8.87	0.0060
Belinostat	69.25	30.48	0.3873	20.43	2.30	0.0186
Uracil mustard	60.47	6.94	0.4625	20.83	8.24	0.0062
Vemurafenib	94.41	25.77	0.0648	21.70	8.76	0.0004
Tretinoin	64.65	18.97	0.3964	22.00	12.64	0.0078
Celecoxib	74.46	9.63	0.1548	22.26	10.58	0.0222
Letrozole	74.09	18.63	0.1597	23.05	12.78	0.0084
Idelalisib	107.33	53.60	0.0770	23.33	7.66	0.0228
Sorafenib	109.77	53.11	0.0654	25.27	2.15	0.0245
Trifluridine	64.28	10.40	0.3301	25.49	14.22	0.0106
Olaparib	48.91	7.80	0.8805	26.22	17.61	0.0314
Altretamine	76.06	22.92	0.1644	26.78	14.84	0.0119
Bleomycin sulfate	65.36	17.57	0.5975	27.10	18.76	0.0005
Chlorambucil	97.00	12.98	0.0160	27.79	0.10	0.0282
Fludarabine phosphate	96.06	15.76	0.0206	27.98	3.86	0.0288
Pomalidomide	51.82	6.63	0.9596	29.34	13.03	0.0132
Pentostatin	43.41	12.50	0.5703	30.14	15.30	0.0153
Methotrexate	53.39	12.50	0.8898	30.50	5.91	0.0337
Uridine triacetate	99.62	32.52	0.0426	30.88	13.82	0.0379
Lenalidomide	68.22	20.01	0.2987	31.27	16.56	0.0174
Niraparib hydrochloride	113.25	43.05	0.0302	32.44	3.89	0.0372
Mercaptopurine	73.09	27.40	0.2590	33.15	14.99	0.0186
Thioguanine	97.80	28.93	0.0334	34.67	4.58	0.0156
Temsirolimus	77.37	12.41	0.1698	35.58	10.35	0.0004
Cisplatin	65.38	15.48	0.3377	36.09	16.35	0.0241
Dactinomycin	59.55	7.40	0.8521	37.23	9.33	0.0018
Cytarabine hydrochloride	55.71	16.07	0.7579	37.56	16.97	0.0274
Ribociclib	88.45	28.22	0.0840	39.99	2.27	0.0572
Bendamustine	145.72	27.91	0.0008	40.18	6.22	0.0589
Mechlorethamine	62.84	16.37	0.4362	40.32	19.67	0.0368

Drug	Mean	SE	p-value	Mean	SE	p-value
Carboplatin	109.45	5.44	0.0031	40.56	14.13	0.0655
Axitinib	103.77	38.65	0.0449	41.06	0.66	0.0607
Bortezomib	97.36	24.94	0.0321	42.01	19.11	0.0767
Thiotepa	51.13	18.88	0.9964	43.00	22.76	0.0499
Palbociclib	81.45	10.52	0.0963	44.99	9.60	0.0010
Thalidomide	54.46	10.16	0.8029	45.05	25.84	0.0643
Ponatinib	107.82	10.52	0.0033	45.06	5.14	0.0007
Erlotinib hydrochloride	63.49	18.80	0.4792	45.14	16.31	0.0871
Oxaliplatin	103.68	11.83	0.0075	45.40	14.28	0.0859
Docetaxel	68.48	6.88	0.3887	45.58	3.04	0.0006
Methoxsalen	54.15	12.03	0.8268	46.23	24.06	0.0646
Cobimetinib	55.14	27.27	0.9171	47.23	28.19	0.0120
Aminolevulinic acid	60.95	6.66	0.4387	47.24	21.67	0.0630
Vismodegib	123.78	30.64	0.0054	47.92	16.34	0.1014
Topotecan hydrochloride	127.64	41.48	0.0101	48.60	5.51	0.0949
Pemetrexed, Disodium	69.55	2.43	0.2337	49.17	17.36	0.1101
Pipobroman	55.94	15.97	0.7741	49.67	2.58	0.0998
Cabozantinib	91.36	12.52	0.0242	49.83	11.99	0.0008
Imiquimod	84.91	18.72	0.1029	51.78	14.43	0.0037
Gemcitabine	109.45	23.35	0.0059	51.86	27.93	0.1052
Ifosfamide	66.43	4.50	0.2269	51.99	21.89	0.0864
Irinotecan hydrochloride	83.32	4.05	0.0585	52.01	6.67	0.0017
Rucaparib phosphate	103.26	24.46	0.0182	52.38	8.59	0.1196
Pazopanib hydrochloride	65.83	4.23	0.7443	53.88	8.68	0.0016
Dacarbazine	163.44	23.53	0.0001	54.42	22.83	0.1039
Estramustine phosphate	68.12	11.44	0.4327	54.93	18.37	0.0164
Megestrol acetate	102.06	24.26	0.0201	55.46	22.50	0.1648
Melphalan hydrochloride	115.20	1.93	0.0016	55.52	11.15	0.1451
Tamoxifen citrate	74.55	24.73	0.3449	56.36	29.88	0.0319
Omacetaxine	92.97	21.80	0.0552	56.46	11.47	0.0047
Everolimus	56.16	11.45	0.9448	56.66	6.42	0.0110
Lapatinib	55.84	21.64	0.9396	57.19	15.09	0.0169
Venetoclax	110.18	5.85	0.0017	58.29	16.43	0.0099
Lenvatinib	124.12	2.27	0.0007	58.36	32.46	0.2194
Temozolomide	92.02	9.37	0.0087	60.45	16.46	0.1286
Lomustine	57.68	12.56	0.6359	60.82	29.82	0.1856
Cladribine	97.04	12.58	0.0062	61.21	20.60	0.1493
Decitabine	115.40	21.14	0.0024	61.38	16.22	0.1359
Erismodegib	92.04	40.25	0.0626	62.16	18.05	0.0040
Exemestane	96.46	17.26	0.0114	62.43	20.58	0.1610
Vorinostat	140.89	11.51	0.0000	62.80	24.34	0.1805
Paclitaxel	68.75	7.90	0.3834	62.85	9.20	0.0086
Vinorelbine tartrate	60.78	3.56	0.7706	63.88	3.88	0.0071
Regorafenib	74.30	25.90	0.3632	64.22	12.72	0.0307
Fluorouracil	83.45	37.59	0.1842	64.51	15.92	0.1656
Arsenic trioxide	119.92	12.76	0.0004	65.23	26.52	0.2185
Dabrafenib mesylate	114.96	7.27	0.0010	66.14	27.12	0.0610

Drug	Mean	SE	p-value	Mean	SE	p-value
Ixabepilone	75.36	6.41	0.1764	66.17	32.04	0.0933
Plerixafor	77.44	16.90	0.2028	66.27	25.02	0.0525
Azacitidine	136.35	14.25	0.0001	67.08	19.92	0.2108
Anastrozole	85.42	5.94	0.0172	67.12	23.48	0.2273
Cyclophosphamide	73.29	11.29	0.1195	67.95	17.12	0.2106
Hydroxyurea	90.96	27.83	0.0568	69.12	16.04	0.2222
Floxuridine	62.40	14.30	0.4343	69.52	51.57	0.4179
Trametinib	65.86	21.06	0.6028	69.61	19.37	0.0467
Pralatrexate	72.81	31.93	0.3153	70.98	6.60	0.3172
Osimertinib	78.61	20.66	0.2123	72.02	54.97	0.3076
Amifostine	106.90	22.39	0.0139	72.88	20.04	0.0697
Streptozocin	114.96	26.89	0.0055	74.16	21.16	0.3237
Cabazitaxel	87.69	15.33	0.0605	76.53	12.46	0.0628
Zoledronic acid	51.39	10.20	0.6732	77.25	5.39	0.0479
Procarbazine	115.10	34.60	0.0133	77.43	15.01	0.3587
Triethylenemelamine	54.61	10.97	0.8549	79.17	33.63	0.2470
Carmustine	83.97	16.63	0.0465	80.53	23.99	0.4652
Vincristine sulfate	78.41	2.42	0.1103	80.72	21.01	0.1629
Fulvestrant	80.46	21.57	0.1860	83.22	30.71	0.2963
Allopurinol	108.69	40.74	0.0378	86.60	21.97	0.6044
Bosutinib	95.63	26.56	0.0281	86.88	55.49	0.1253
Etoposide	102.81	8.99	0.0055	87.25	15.77	0.2465
Enzalutamide	73.87	26.47	0.3802	91.54	4.70	0.3893
Carfilzomib	43.78	11.96	0.3491	92.38	32.29	0.5477
Busulfan	53.11	10.92	0.8850	93.11	20.47	0.7855
Alectinib	51.68	8.86	0.2841	93.37	34.10	0.5642
Valrubicin	48.42	6.45	0.5039	94.33	17.34	0.4869
Nilotinib	43.89	4.80	0.7537	95.03	45.99	0.4474
Ixazomib citrate	93.03	14.03	0.0789	97.69	46.66	0.3669
Sirolimus	46.20	15.14	0.4658	101.05	15.19	0.7794
Daunorubicin	70.06	9.43	0.3443	103.79	46.47	0.9578
Vandetanib	96.11	30.67	0.0788	104.60	97.73	0.9911
Vinblastine sulfate	79.02	15.06	0.1586	109.81	16.22	0.7601
Abiraterone	63.92	8.91	0.6105	116.20	23.79	0.5374
Romidepsin	66.63	6.35	0.1377	134.18	16.87	0.7031
Sunitinib	62.87	36.85	0.6078	200.50	25.70	0.0097
Doxorubicin	90.64	6.81	0.0238	222.83	27.38	0.0002
Epirubicin hydrochloride	92.22	8.06	0.0207	405.48	63.93	0.0000
Idarubicin hydrochloride	66.27	23.03	0.6012	5341.22	3598.06	0.0182

Table 4.2. Results of drug-screening on MRSA infection in HeLa cells. The effect of 133 FDA-approved drugs was tested in HeLa cells after MRSA infection (MOI 100, 6h). The percentage of cell number was normalized to percentage of uninfected cells. The percentage of HeLa cells containing *S. aureus* USA300-GFP was normalized to the untreated infected cells. Data show mean, standard error and p-value of three biological replicates.

4.2.3. Ibrutinib halts the cell invasion and intracellular survival of *S. aureus*

To test whether the observed effect of Ibrutinib on host cell infection by *S. aureus* was due to the host-pathway inhibition or a direct effect on bacterial growth, we measured bacterial growth in the presence and absence of Ibrutinib as well as other controls. We did not find any significant differences in *S. aureus* growth in the presence of Ibrutinib when compared to the growth on rich media only, indicating that the previous observations resulted from a host-directed effect (Fig. 4.5).

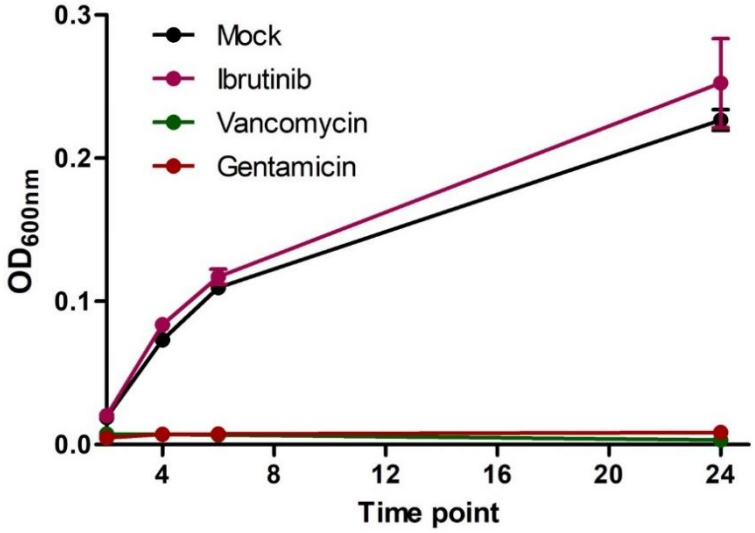

Figure 4.5. Ibrutinib does not have a direct effect on *S. aureus* growth. *S. aureus* USA300 was grown in 96-wells plate in the presence of DMSO (Mock), Ibrutinib, vancomycin or gentamicin. Absorbance (OD_{600nm}) was measured at 2, 4, 6 and 24 hours to produce the growth curve. Data are expressed as means ± SE of three different experiments performed in triplicates.

To validate cell viability readings based on mCherry expression, I measured markers that are activated upon cell death (Annexin-FITC and Propidium Iodide) and found again a significant increase of cell viability in *S. aureus*-infected cells that were treated with Ibrutinib in comparison to the untreated cells (Fig 4.6A). Additionally, we then measured the intracellular survival of *S. aureus* in the

presence of Ibrutinib (10 μM) and found that recovery of intracellular *S. aureus* was significantly lowered after both two and six hours of infection in the presence of Ibrutinib (Fig 4.6B). Specifically, the reduction of intracellular MRSA was more pronounced at early times of infection (two hours), suggesting that the drug's effect is particularly important for cell invasion and/or internalization.

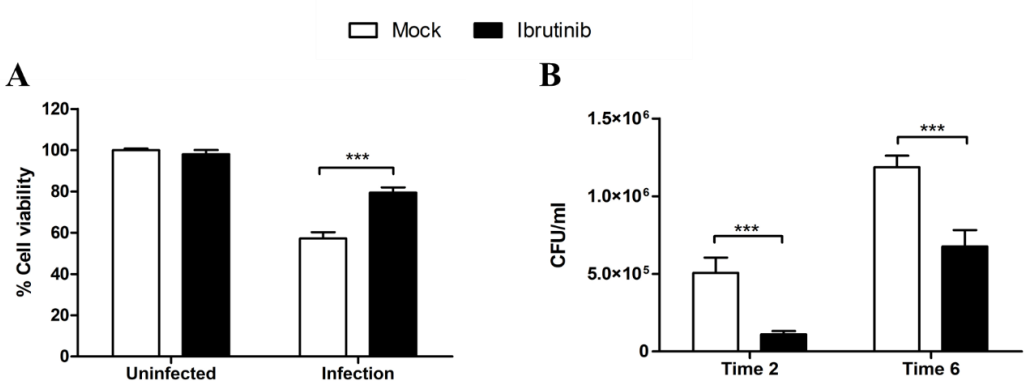

Figure 4.6. Ibrutinib treatment during *S. aureus* infection increases host cell viability whereas intracellular bacterial survival is hampered. HeLa cells were infected with *S. aureus* USA300 (MOI 100; 6 hours) in the presence of DMSO (Mock) or Ibrutinib. (A) Host cell viability was quantified by flow cytometry, using a double annexin V-FITC and PI staining. Cell viability was normalized by the percentage of uninfected and untreated cells. (B) Intracellular MRSA survival was quantified by CFU counting after 2 and 6 hours of infection. Data are expressed as means ± SE of at least three different experiments performed in duplicates and Student´s t-tests were performed to validate statistical significance across conditions. p-value ≤0.001 (***).

Cytoplasmic replication of *S. aureus* upon phagosomal escape is considered one of the last steps of a successful cell invasion (Grosz, Kolter, Paprotka, A. Winkler, *et al.*, 2014). Consequently, the quantification of the number of cytosolic bacteria per cell could be considered an alternative way to evaluate the intracellular proliferation of *S. aureus*. Therefore, we quantified the number of cytosolic bacteria per cell using a phagosomal escape reporter C-terminal cell wall-targeting domain (CWT) of lysostaphin (Bravo-Santano *et al.*, 2018) and found a four-fold reduction in bacterial numbers in the cytosol in the presence of Ibrutinib (Fig. 4.7) .

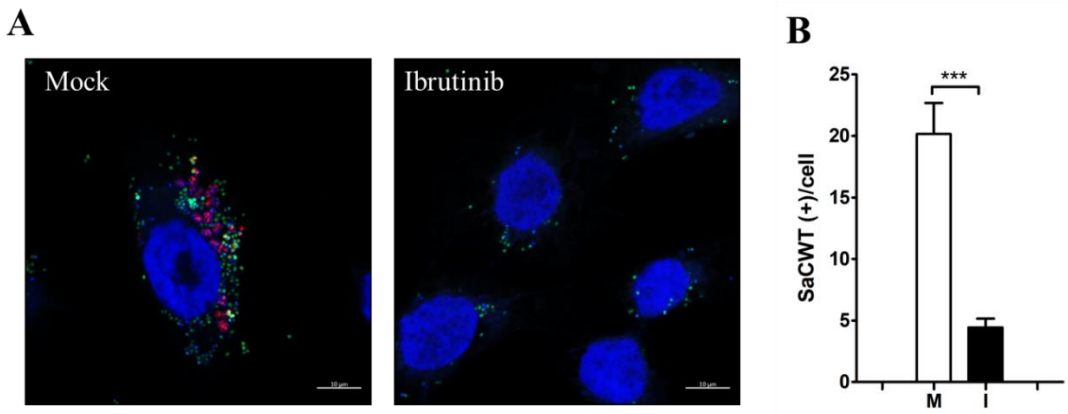

Figure 4.7. Phagosomal escape of *S. aureus* USA300-GFP to the host cytosol under Ibrutinib treatment. HeLa cells expressing the phagosomal escape reporter mCherry-SaCWT were infected with *S. aureus* USA300-GFP (MOI 100; 6 hours) in the presence or absence of Ibrutinib. (A) Images were taken using confocal microscopy. DAPI was employed for nucleus staining. Bar indicates 10 µm. (B) Quantification of number bacteria positively stained with mCherry-SaCWT per cell. Data are expressed as means ± SE of three different experiments and Student´s t-tests were performed to validate statistical significance across conditions. p-value ≤0.001 (***). M, Mock; I, Ibrutinib. Microscopy picture was taken by Anna Klose and Elizabeth Boudriot during their placement in our laboratory.

4.2.4. Ibrutinib's effect on *S. aureus* intracellular infection is likely to be BTK-independent

Ibrutinib is a potent inhibitor of the Bruton's tyrosine kinase (BTK) pathway in B cells. However, BTK is mainly expressed in cells of the immune system (Genevier et al. 1994) and a number of alternative protein kinases that are targeted by Ibrutinib have been recently identified, e.g. EGFR, ITK or TEC (Berglöf *et al.*, 2015).

To test the putative role of BTK in our model, we evaluated the effect on intracellular *S. aureus* survival of another two BTK inhibitors, Acalabrutinib and ONO-4059 (Wu *et al.*, 2016). We did not detect significant differences on host cell viability or bacterial intracellular survival for these compounds (Fig. 4.8),

suggesting a BTK-independent mechanism of action due to an off-target effect of Ibrutinib in epithelial-like cells.

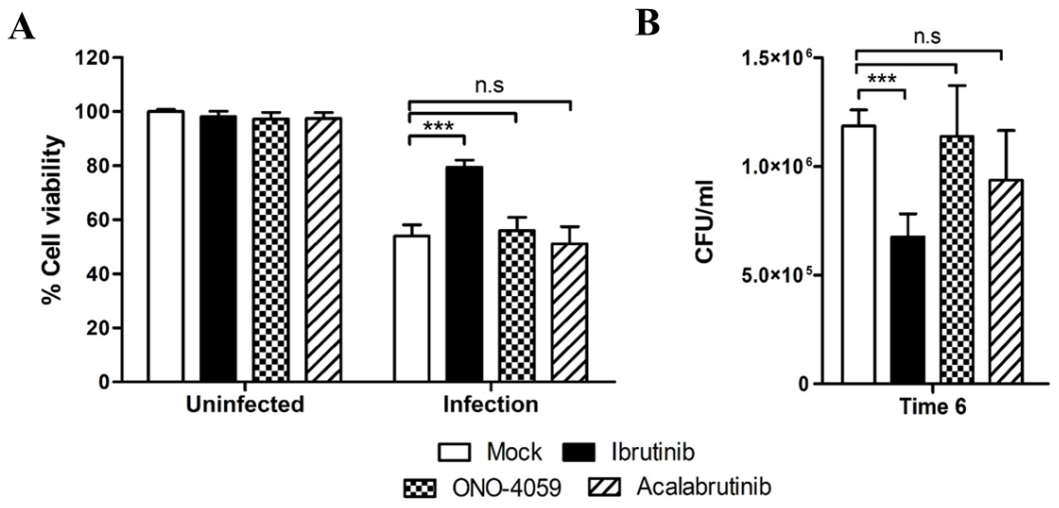

Figure 4.8. Effect of Btk's inhibitors – Ibrutinib, Acalabrutinib and ONO-4059 – on *S. aureus* infection in HeLa cells. HeLa cells were infected with *S. aureus* USA300 (MOI 100; 6 hours) in the presence of DMSO (Mock), Ibrutinib, Acalabrutinib or ONO-4059. (A) Host cell viability was quantified by flow cytometry, using a double annexin V-FITC and PI staining. Cell viability was normalized by the percentage of uninfected and untreated cells. (B) Intracellular MRSA survival was quantified by CFU counting after 6 hours of infection. Data are expressed as means ± SE of three different experiments performed in duplicates and Student´s t-tests were performed to validate statistical significance across conditions. p-value ≤0.001 (***), (n.s) no statistical significance.

4.2.5. Phosphoproteomics of *S. aureus*-infected cells in response to Ibrutinib identifies candidate pathways

To elucidate the putative mechanism of action of Ibrutinib in our model, I performed a phosphoproteomics analyses in *S. aureus*-infected cells in presence and absence of Ibrutinib.

Overall, I detected approximately 10,000 hits with four different types of modification: phosphorylation in the tyrosine kinases (P(Y)), phosphorylation in serine/threonine kinases (P(ST)), phosphorylation in both sites at the same time (P(Y)+P(ST)) or oxidation (O). Phosphorylation in the serine/threonine kinase sites

was the most common modification detected in our study, representing the 88% of the total modified peptides (Fig. 4.9).

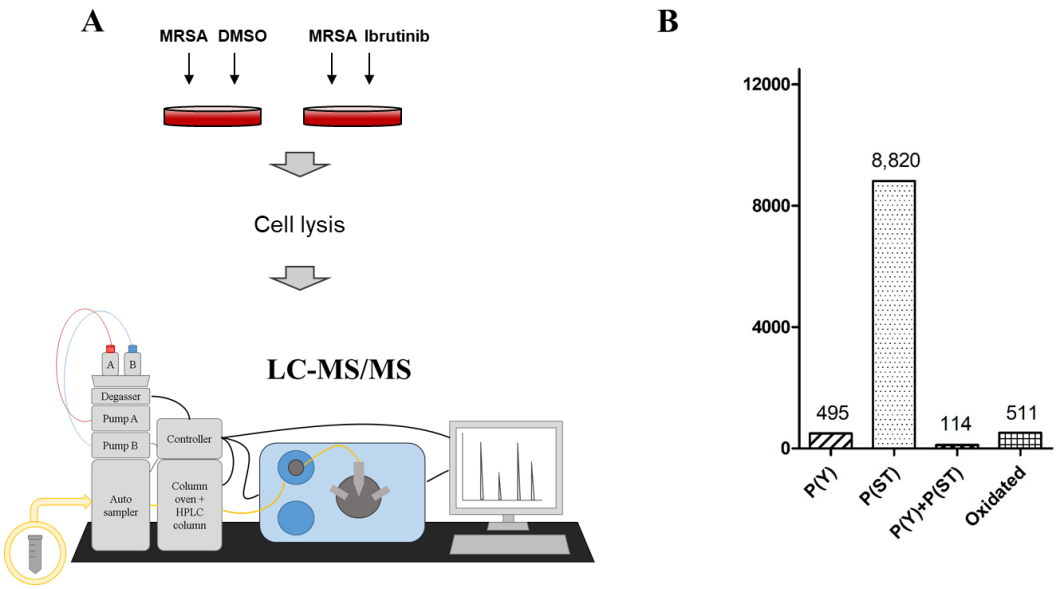

Figure 4.9. Overview of the phosphoproteome analysis and peptide modifications detected. (A) HeLa cells were infected with *S. aureus* USA300 in the presence of DMSO (Mock) or Ibrutinib. Cells were lysed after 6 hours of infection and samples were analysed by LC-MS/MS to identify phosphoproteomics changes. (B) Total number of peptides detected by LC-MS/MS classified by the different modifications that were detected: P(Y), phosphotyrosines; P(ST) phosphorserines/threonines; P(Y)+P(ST), phosphotyrosine phosphorserines/threonines; Oxidated, peptides with residues subjected to oxidation.

For the phosphoproteomics analysis, we included two biological replicates for each condition – Mock (DMSO) and Ibrutinib – and samples were also run twice through the LC-MS to have two additional technical replicates. Overall, we found that the phosphoproteome of MRSA-infected cells was clearly affected by the treatment with Ibrutinib (Fig. 4.10A). Moreover, although some variability was observed across samples, biological and technical replicates were highly reproducible (Fig. 4.10B).

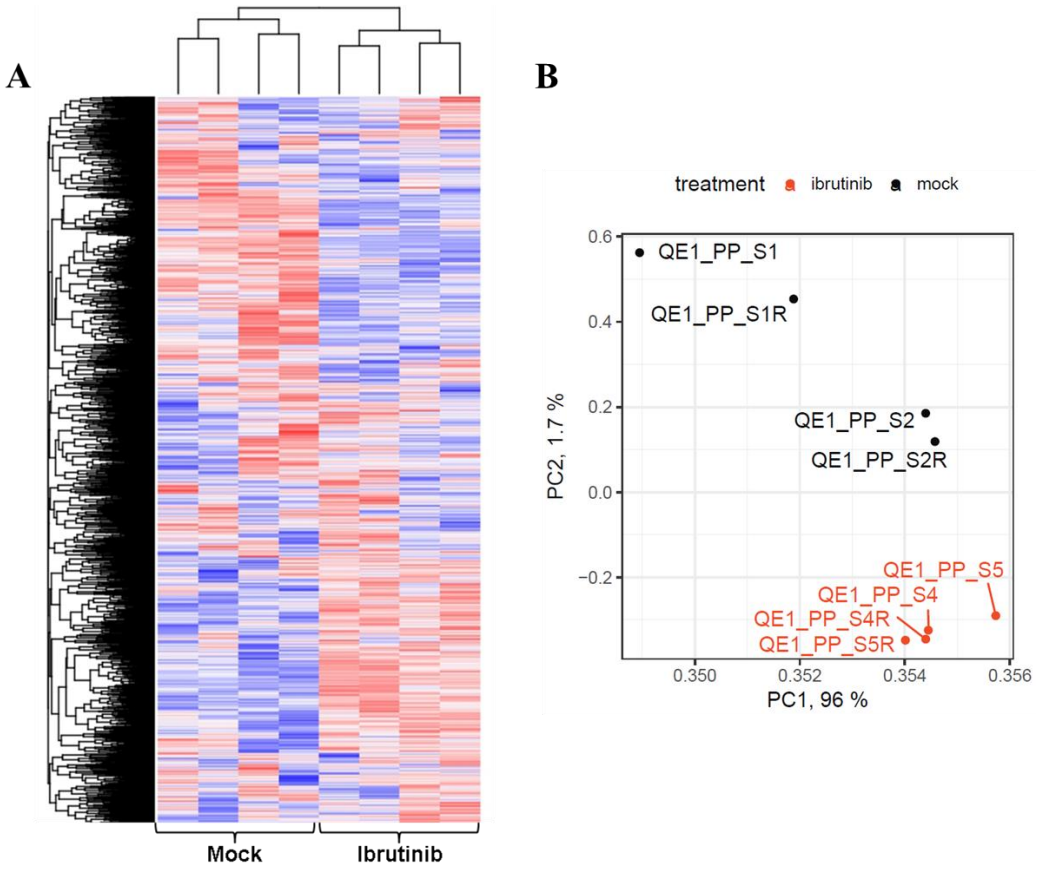

Figure 4.10. Differences of phosphopeptides levels in *S. aureus*-infected cells under Ibrutinib treatment. (A) Relative values of every phosphopeptide detected through LC-MS/MS was plotted as a Heat-map. Each treatment is comprised by four replicates (two technicals and two biologicals) of each treatment: mock (DMSO) and Ibrutinib. (B) Principal Component Analyses (PCA) of the phosphoproteomics data in MRSA-infected cells. Untreated samples are represented in black whereas Ibrutinib-treated samples are displayed in red. Both plots were provided by Dr. Pedro Cutillas from Barts Cancer Institute facilities.

Ibrutinib is an inhibitor of tyrosine (Y) kinases; however, as an off-target effect of this drug may play a role during *S. aureus* infection, we also included peptides with phosphoserine/threonine (S/T) sites in our analyses. We focused our analyses on the most significant hits, and 34 proteins were identified when applying a strict cut-off (log2 fold change ≤ -2 and log10 p-value ≥ 2; Fig. 4.11).

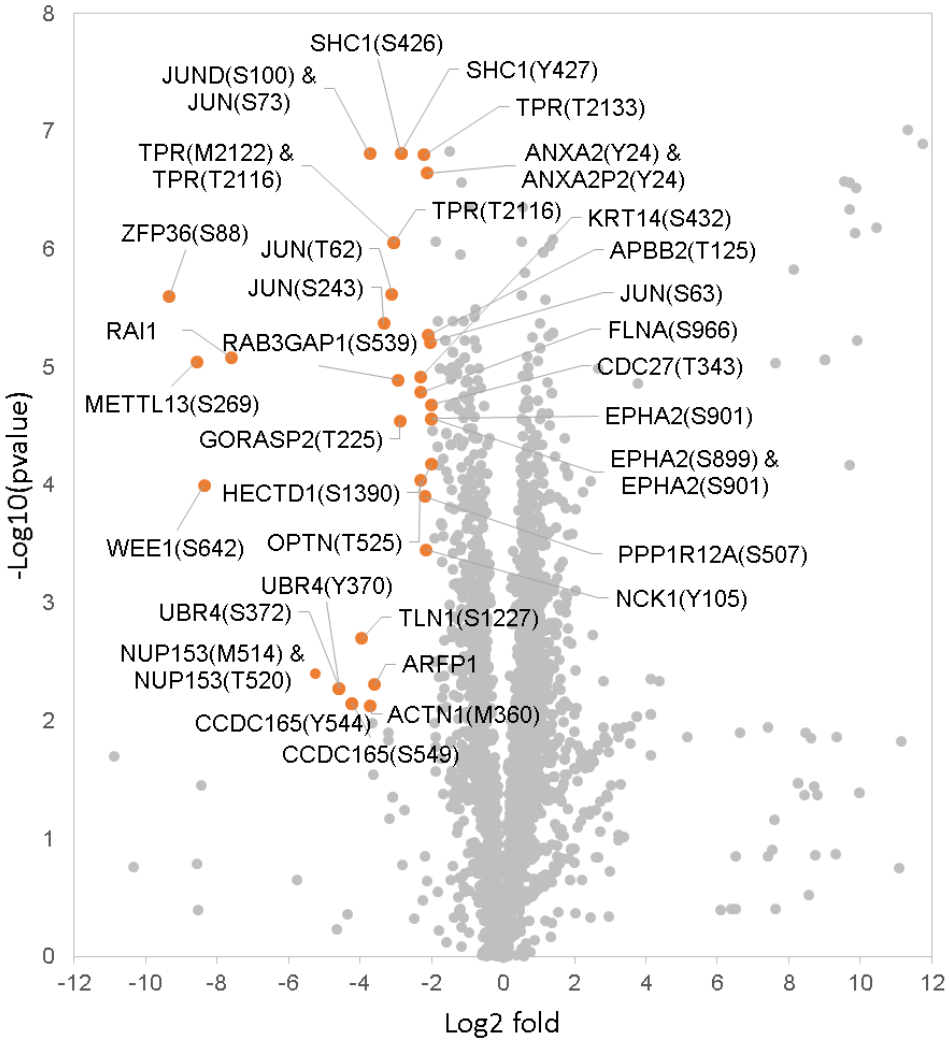

Figure 4.11. Phosphoproteome of *S. aureus*-infected HeLa cells under Ibrutinib treatment. Log2 Fold change is plotted against the Log10 p-value of each phosphopeptide under Ibrutinib treatment in relation to the untreated control. Orange dots comprise phosphopeptides whose Log2 fold was lower than -2 and Log10 p-value was higher than 2.

Of these, several proteins could control host pathways that could be crucial during cell infection. Our hits included several phosphopeptides of the JUN and JUND transcriptional factors of the AP-1 complex, which are at the crossroad of different signalling pathways required for proliferation, survival or apoptosis (Hernandez *et al.*, 2008; Meng and Xia, 2011). In addition, we also identified ANXA2, a multifaceted protein that is involved in intracellular membrane repair, among many other processes (Hajjar, 2015); RAB3GAP1 modulates autophagosomal biogenesis

(Spang *et al.*, 2014) and FLNA have different roles on cytoskeleton remodelling (Ohta, Hartwig and Stossel, 2006). Finally, EPHA2 stimulates the MAP/ERK kinase signalling cascade through SHC1 (Pratt and Kinch, 2002), which controls the expression of several genes involved in cell cycle.

To further explore the phosphoproteome of *S. aureus*-infected cells under Ibrutinib treatment, I performed a functional pathway analysis by using DAVID Bioinformatics Resources 6.8 (Huang, Sherman and Lempicki, 2009). For this, phosphopeptides with p-values <0.05 and log2 fold change ≤ -1.1 were selected. As a result, 158 phosphopeptides were analysed to investigate the KEGG pathways affected by the treatment, which revealed that Ibrutinib mainly influences five cellular pathways in the infected host cells: focal adhesion, ERBB and MAPK signalling pathway, adherens junction and the regulation of actin cytoskeleton (Table 4.1).

Cellular pathway	Number of genes	p-value	Benjamini
Focal adhesion	13	5.10E-08	3.80E-06
ErbB signaling pathway	10	2.10E-08	3.10E-06
Adherens junction	7	1.90E-05	9.30E-04
MAPK signaling pathway	11	2.60E-05	1.00E-03
Regulation of actin cytoeskeleton	10	3.70E-05	1.10E-03

Table 4.3. Cellular pathways affected by Ibrutinib treatment during MRSA infection. KEGG pathways were analysed employing DAVID Bioinformatics Resources 6.8. Only cellular pathways that were statistically significant after Benjamini correction were included (p-value < 0.01).

Twenty proteins were commonly implicated in these five cellular pathways (Fig. 4.12 and Table 4.2), from which we identified several kinases in our study that might be the direct target of Ibrutinib during MRSA infection in HeLa cells, e.g. EPHA2, EGFR or ERBB2.

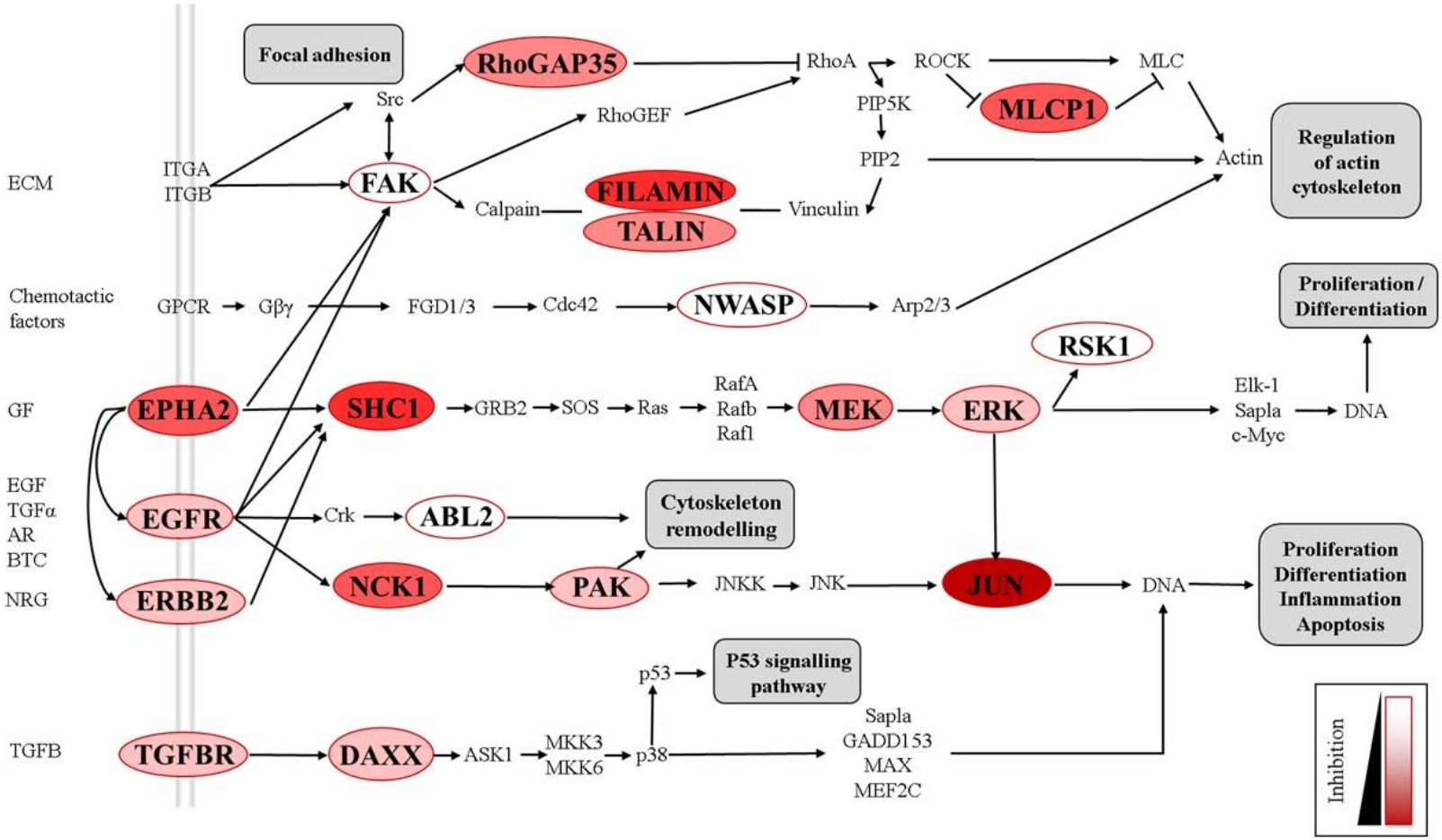

Figure 4.12. Effects of Ibrutinib on the phosphoproteome of *S. aureus*-infected HeLa cells. The genes for which we detected a modification in their phosphorylation levels are displayed in capitals and surrounded by a red line. The level of inhibition of protein phosphorylation under Ibrutinib treatment is represented by the intensity of the red-colour within the circle as shown by the legend. Different pathways that are downstream affected by the inhibition of these genes are illustrated in grey-squares.

Gene	Description	Modification	Log2Fold	p-value
ABL2	Tyrosine-protein kinase ABL2 protein.	ABL2(T938)	-1.187	0.0039
ARHGAP35	Rho GTPase-activating protein 35.	ARHGAP35 (S1153)	-1.588	1.42E-04
DAXX	Death domain-associated protein 6.	DAXX(S495)	-1.304	0.0138
EGFR	Epidermal growth factor receptor protein.	EGFR(S695)	-1.422	0.0076
ERBB2	Receptor tyrosine-protein kinase erbB-2 protein.	ERBB2(T694)	-1.364	2.27E-04
EPHA2	Ephrin type-A receptor 2 protein.	EPHA2(S899); EPHA2(S901)	-2.000	5.57E-04
		EPHA2(S901)	-2.000	5.57E-04
FLNA	Filamin A protein.	FLNA(T1739)	-3.122	1.23E-05
		FLNA(S966)	-2.293	1.88E-04
FLNB	Filamin B protein	FLNB(S2487)	-1.499	0.0013
		FLNB(S2113)	-1.168	3.42E-04
JUN	Transcription factor AP-1 protein	JUN(S243)	-3.337	3.83E-05
		JUN(T62)	-3.103	6.84E-08
		JUN(S63)	-2.037	4.56E-05
MAP2K2	Mitogen-activated protein kinase kinase 2	MAP2K2(T230); MAP2K1(T226)	-1.629	0.0092
MAPK3	MAPK3/ERK1 protein.	MAPK3(T198); MAPK3(Y204)	-1.390	1.77E-06
		MAPK3(Y204)	-1.390	1.77E-06
NCK1	Cytoplasmic protein NCK1	NCK1(Y105)	-2.159	5.82E-04
PAK2	Serine/Threonine protein kinase PAK 2	PAK2(T21)	-1.378	0.0074
PPP1R12A	Protein phosphatase 1 regulatory subunit 12A	PPP1R12A(T305)	-1.531	1.72E-04
		PPP1R12A(S304)	-1.443	6.26E-05
		PPP1R12A(S299)	-1.294	0.0028
		PPP1R12A(S507)	-2.171	5.94E-04
PTK2	Focal adhesion kinase 1 protein	PTK2(S910)	-1.148	7.21E-04
RPS6KA1	Ribosomal protein S6 kinase alpha-1	RPS6KA1(S732)	-1.189	1.02E-06
SHC1	SHC-transforming protein 1	SHC1(S426)	-2.853	9.71E-06
		SHC1(Y427)	-2.853	9.71E-06
TGFBR2	TGF-beta receptor type-2 protein	TGFBR2(S352)	-1.453	2.48E-04
TLN1	Talin 1 protein	TLN1(S1225)	-1.595	4.09E-05
		TLN1(S1227)	-3.955	0.0439
WASL	Neural Wiskott-Aldrich syndrome protein	WASL(Y256)	-1.112	3.16E-05

Table 4.4. List of genes involved in KEGG pathways affected by Ibrutinib treatment during *S. aureus* infection.

Lastly, a kinase substrate enrichment analysis (KSEA, Casado et al. 2013) was performed. KSEA is a powerful tool to estimate kinase activity based on the collective phosphorylation status of its substrates. Using this analysis, we identified several kinases that belong to MEK/ERK pathway (MEK2, ERK1/2/7, RSK2 and p90RSK) and three c-Jun N-Terminal kinases (JNK1/2/3; Fig. 4.13), which is showing the importance of the MEK/ERK/c-JUN signalling for intracellular *S. aureus* survival and proliferation. Conversely, none of our analysis flagged up BTK as a target in our model, further pointing towards a BTK-independent mechanism of action for Ibrutinib during infection of epithelial-like cells by *S. aureus*.

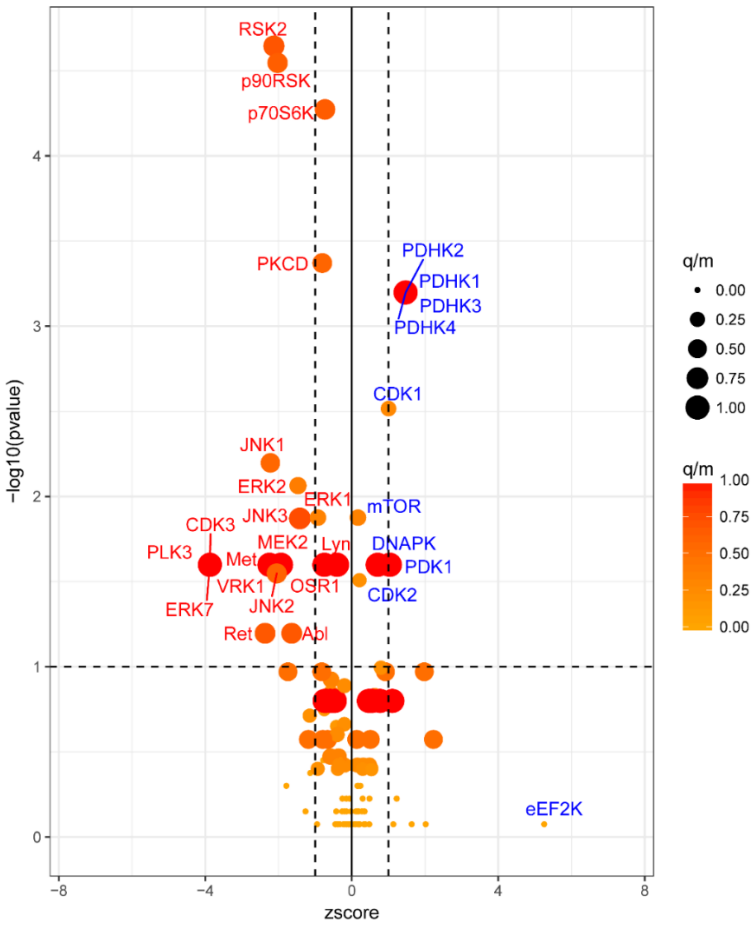

Figure 4.13. KSEA analysis of the phosphoproteomics results. Size and color intensity of each dot is proportional to the ratio of Ibrutinib-treated samples compared to untreated samples. The larger and darker the dot is, bigger was the observed effect in Ibrutinib-treated samples. Kinases that were activated or inhibited in the presence of Ibrutinib are highlighted in blue and red, respectively. KSEA plot was provided by Dr. Pedro Cutillas from Barts Cancer Institute facilities.

4.2.6. Downstream analysis reveals the importance of EPHA2 for staphylococcal invasion

To further characterize the host cell response to intracellular *S. aureus*, I investigated expression and phosphorylation levels of selected targets by Western blot analysis.

Interestingly, N-WASP was identified as a potential target of the Ibrutinib effect on MRSA-infected cells. *S. aureus* triggers actin comet-tails through N-WASP phosphorylation (Schröder *et al.*, 2006). In addition, the intracellular pathogen *Shigella flexneri* promotes Btk expression and N-WASP phosphorylation for its intracellular dissemination (Dragoi, Talman and Agaisse, 2013).

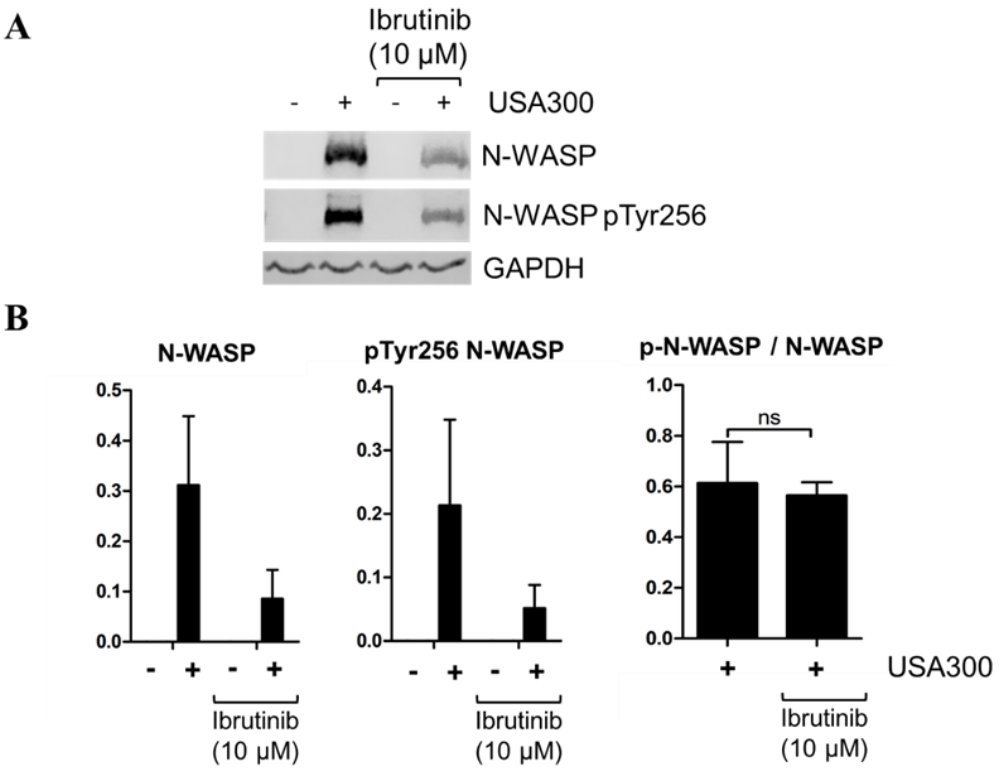

Figure 4.14. Phosphorylation of N-WASP protein was not significantly affected by Ibrutinib treatment. HeLa cells were infected with *S. aureus* USA300 (MOI 100; 6 hours) in the presence or absence of Ibrutinib. (A) Protein lysates were analysed by Western-blot against N-WASP and phospho-N-WASP. GAPDH antibody was employed as loading control. (B) Quantification of N-WASP and phospho-N-WASP levels observed by Western-blot. Data were normalized to GAPDH levels for each condition and are expressed as means ± SE of two different experiments. Student´s t-tests were performed to validate statistical significance across conditions. (n.s) no statistical significance. Western-Blot was performed by Helen Stölting during her placement in our laboratory.

Accordingly, the total protein levels of N-WASP were markedly increased after *S. aureus* infection, and these levels were lowered in infected cells treated with Ibrutinib (Fig 4.14A). However, the phospho-N-WASP / N-WASP ratio was not significantly affected by Ibrutinib in MRSA-infected cells (Fig. 4.14B), suggesting that the effect of Ibrutinib on N-WASP phosphorylation levels is indirect, and ruling out again an implication of BTK.

In concordance with our phosphoproteomics data, we observed that the phosphorylation levels of c-JUN were downregulated in infected cells treated with

Ibrutinib (Fig. 4.15). Furthermore, basal levels of c-JUN remained stable among the three conditions, which indicates that Ibrutinib is either directly controlling the phosphorylation of this protein or is inhibiting another kinase involved in the activation of c-JUN phosphorylation (Fig. 4.15).

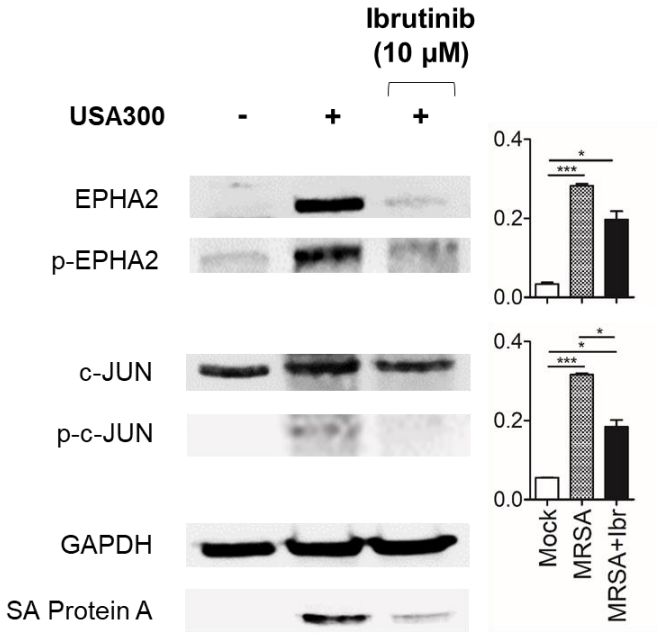

Figure 4.15. Phosphorylation of EPHA2, c-JUN and ERK1/2 proteins in *S. aureus*-infected cells treated with Ibrutinib. HeLa cells were infected with *S. aureus* USA300 (MOI 100; 6 hours) in the presence or absence of Ibrutinib. Protein lysates were analysed by Western-blot against EPHA2, p-EPHA2, c-JUN, p-c-JUN, ERK1/2 and p-ERK1/2. GAPDH antibody was employed as loading control. Protein A from *S. aureus* was also detected and included as an internal control of the *S. aureus* infection.

The basal levels of EPHA2 protein were markedly increased in *S. aureus*-infected cells compared to the uninfected control and were downregulated by Ibrutinib treatment (Fig. 4.15). Interestingly, EPHA2 has been shown to be important for other models of intracellular infections (Du Toit 2018). As inhibitors of Ephrin receptors such as ALW-II-41-27 exhibit cross reactivity with a number of EPHA and EPHB receptors (e.g. EPHA2/5/8 and EPHB1/2/3) (Choi *et al.*, 2009), we decided to confirm the role of EPHA2 during intracellular *S. aureus* infection in an

EphA2 knockout cell lines derived from HEK293T (Chen *et al.*, 2018). We quantified the host cell viability and intracellular bacterial survival and – in agreement with our previous results – we found that host cell viability was significantly higher in EphA2 knockout cell lines after six hours of *S. aureus* infection when compared to the wild type. Moreover, intracellular bacterial survival was significantly reduced after two and six hours of infection in EphA2 knockout cell lines (Fig. 4.16), suggesting that the EPHA2 receptor is important for the successful internalization of *S. aureus* inside host cells.

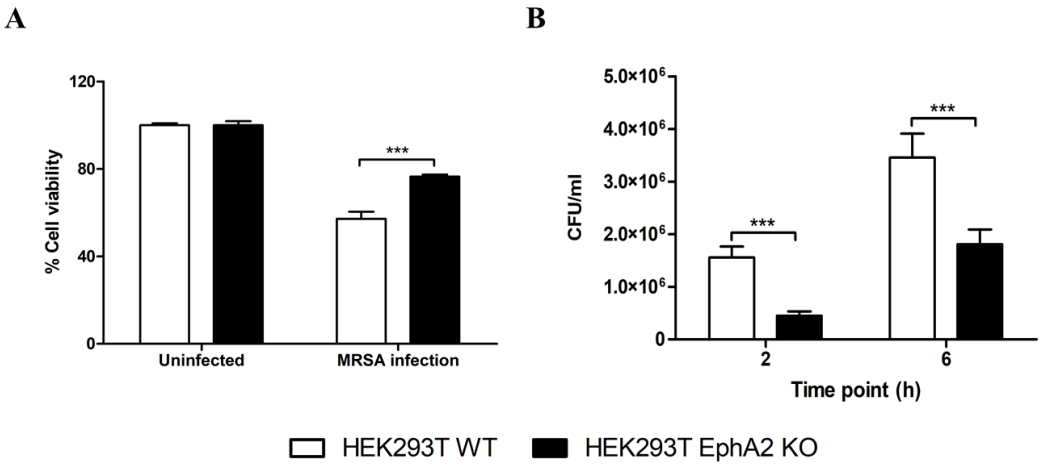

Figure 4.16. The host-membrane receptor EPHA2 plays an important role during intracellular *S. aureus* infection. HEK-293T wild type and EphA2 knockout cells were infected with *S. aureus* USA300 (MOI 100) for 6 hours. (A) Host cell viability was quantified by flow cytometry, using a double annexin V-FITC and PI staining. Cell viability was normalized by the percentage of uninfected cells. (B) Intracellular MRSA survival was quantified by CFU counting after 2 and 6 hours of infection. Data are expressed as means ± SE of at least three different experiments performed in duplicates and Student´s t-tests were performed to validate statistical significance across conditions. p-value ≤0.001 (***).

4.3. DISCUSSION

4.3.1. Tyrosine kinase inhibitors as promising host-directed therapeutics for intracellular MRSA infection

In this study, we identified three potential candidates that may contribute to the clearance of intracellular MRSA infection (Ibrutinib, Dasatinib and Crizotinib) from a pool of 140 host-directed drugs. All three are host tyrosine kinase inhibitors, usually employed to treat cancer (D'cruz and Uckun, 2013). For in-depth characterisation, we focused on Ibrutinib, which caused a significant reduction of intracellular bacterial load at early time points of infection. This suggested that the host-pathway inhibited by Ibrutinib could play an important role at early steps of infection, such as bacterial invasion or internalization.

Among all the different host-directed strategies against bacterial and viral pathogens, tyrosine kinase inhibitors have shown potential for clearing intracellular infections and two drugs are already under preclinical trials: Imatinib and Dasatinib (Zumla *et al.*, 2016). Imatinib – an inhibitor of BCR-ABL tyrosine kinase and commercially distributed as Gleevec® – restricts *M. tuberculosis* replication within macrophages and also reduces bacterial load and pathology in mice lungs infected with the same pathogen (Bruns *et al.*, 2012). This drug has also been shown to decrease *Vaccinia* virus titre as well as to prevent virus dissemination *in vivo* models (Schwegmann and Brombacher, 2008). Moreover, a recent study showed that low doses of Imatinib reduce bacterial load of *Franciscella* spp. by inducing myelopoiesis and thus, the stimulation of host-immune responses by Imatinib may promote clearance of several microbial pathogens (Napier *et al.*, 2015). Imatinib

also exhibited promising results in our study but did not meet our stringent cut-off criteria (Table 4.2).

Dasatinib was one of the most promising candidates from our drug screening. After *S. aureus* infection, host cell viability was highly increased whereas intracellular bacterial load was drastically reduced in the presence of this drug. Dasatinib is a tyrosine kinase inhibitor that targets a wide variety of kinases and receptors, including ABL, the SRC family kinases, the receptor tyrosine kinases c-KIT, platelet-derived growth factor receptor (PDGFR), discoidin domain receptor 1 (DDR1), c-FMS, and ephrin receptors (Montero *et al.*, 2011). One of these pathways may be hijacked by MRSA during intracellular infection and therefore, providing a potential mechanism for the significant reduction of intracellular bacterial load during Dasatinib treatment.

Similar to Dasatinib, the host-kinase inhibitors Saracatinib (AZD05030) inhibits Dengue virus replication within Huh7 cells via inhibition of Fyn kinase (de Wispelaere et al. 2013). In our study, we did not test the effect of Saracatinib (AZD05030) on *S. aureus* infection.

4.3.2. The mode of action of Ibrutinib in MRSA-infected epithelial-like cells is BTK-independent

It has previously been shown that some intracellular pathogens are able to manipulate host actin to drive movement through the cytosol and to promote cell to cell spread (Welch and Way, 2014; Lamason and Welch, 2017). Actin maintains tissue integrity by providing structural integrity at cell-to-cell junctions and promotes the formation of membrane extensions during cell migration as well as

intracellular trafficking (Welch and Way, 2014; Lamason and Welch, 2017). Pathogens can trigger host actin-cytoskeleton modifications in different ways: (i) using secreted toxins that directly target actin or its regulators (Aktories *et al.*, 2011), (ii) exploiting actin assembly to promote their invasion (Carabeo, 2011) or (iii) hijacking the host-actin polymerization to induce intracellular or surface-associated motility.

For the pathogen *Shigella flexneri*, it has been suggested that it requires BTK through N-WASP phosphorylation to facilitate actin-based motility and dissemination (Dragoi, Talman and Agaisse, 2013; Valencia-Gallardo, Carayol and Tran Van Nhieu, 2015). Based on these observations and since Ibrutinib is a BTK inhibitor, we initially speculated that this pathway was also important during *S. aureus* infection. However, the levels of both N-WASP and phospho-N-WASP were decreased in MRSA-infected cells after Ibrutinib treatment with the ratio not significantly altered across the different conditions. The reduction in the phosphorylation of N-WASP detected by LC-MS/MS is due to a reduction of basal levels of the protein, probably caused by a reduction of intracellular bacterial load.

As mRNA expression of BTK is usually limited to hematopoietic cells, specifically to B-cells and myeloid cell lines (Genevier et al. 1994), and some off-targets effect of Ibrutinib have already been reported, including non-kinase targets – such as (Lanning *et al.*, 2014) and other kinases targets (Berglöf *et al.*, 2015), we hypothesized that the effect of Ibrutinib is occurring on a BTK-independent manner. To confirm, we tested the effect of two other BTK inhibitors – Acalabrutinib and ONO-4059 – and found no significant effect on either host cell viability or *S. aureus* intracellular survival.

Interestingly, Berglöf et al. reported novel targets of Ibrutinib that are well expressed in epithelial cells, such as Epithelial Growth Factor Receptor family: EGFR, ERBB2 and ERBB4 (Berglöf *et al.*, 2015). Moreover, EGFR and ERBB2 were also desribed to be inhibited by Ibrutinib in B-cells (Honigberg at al. 2010). Hence, these receptors might represent the potential targets of Ibrutinib in epithelial-like cells during MRSA infection.

4.3.3. Ibrutinib alters the phosphoproteome of *S. aureus*-infected cells

Our analysis revealed that the phosphoproteome of *S. aureus*-infected cells was clearly affected by Ibrutinib treatment. Although we found numerous phosphopeptides changes between treatment and control, functional analyses allowed us to identify specific affected pathways. Many of the genes that were inhibited by Ibrutinib are related to cytoskeleton rearrangement of epithelial cells. For example, filamins (FLNA and FLMNB) are actin-binding proteins that stabilize actin filament networks and are important for cell locomotion (Zhou, Hartwig and Akyürek, 2010). In addition, Abl-family kinases are essential regulators of host cytoskeleton. They translate extracellular signals into cytoskeleton rearrangements affecting mainly host cell motility. They are also able to control other signalling molecules, such as Rho and Rac GTPases, to stimulate assembly of protein complexes that activate the formation of actin filaments by the Arp2/3 complex (Bradley and Koleske, 2009). All of these proteins were inhibited under Ibrutinib treatment after *S. aureus* cell infection (Fig. 4.12). Similarly, a treatment with the

inhibitor of actin polymerization cytochalasin D blocks internalization of *S. aureus* in mammalian cells (Murai *et al.*, 1993).

In addition, it has previously been shown that many of the proteins that were inhibited by Ibrutinib in our study play also a role in other bacterial and viral infections. Host Rho-GTPases are involved in actin cytoskeleton remodelling and are manipulated by the HIV-1 envelope protein to facilitate its entry to the host cell (del Real *et al.*, 2004). The MAPK signalling pathway is one of the most prominent host-pathway hijacked by intracellular pathogens (Krachler, Woolery and Orth, 2011). Specifically, *M. tuberculosis* activates host p38-MAPK pathway in order to inhibit CD1 expression and thus, subvert part of the host-immune response (Gagliardi *et al.*, 2009). Additionally, internalization of *S. aureus* has been related to the activation of focal adhesion kinase (FAK) and Src-mediated cortactin phosphorylation (Agerer *et al.*, 2005) and this pathway was also inhibited in the presence of Ibrutinib during MRSA infection. Moreover, a kinase substrate enrichment analyses (KSEA; Fig 4.13) (Casado et al. 2013; Wiredja et al. 2017), identified several ERK and c-JUN protein kinases (JNK) as part of the pathways inhibited by Ibrutinib during MRSA infection. We have previously observed an activation of the host ERK pathway in HeLa cells after *S. aureus* infection (Bravo-Santano N, 2018). In addition, other studies have also shown that internalization of *S. aureus* is impaired when using ERK or JNK inhibitors in fibroblasts and RAW264.7 cells, respectively (Fang *et al.*, 2014; Zhao *et al.*, 2017).

Finally, we have identified several host membrane receptors that could play important roles on *S. aureus* cell infection, e.g. Ephrin type-A receptor 2 (EPHA2), Epidermal growth factor receptors (EGFR or ERBB1 and ERBB2) and transforming growth factor beta receptor (TGFBR). Interestingly, the inhibitor of

the host EGFR, CI-1033, has shown therapeutic efficacy against Vaccinia virus infection (Yang *et al.*, 2005). Moreover, as mentioned above, EGFR and ERBB2 have been previously described as off-targets suspects of the drug Ibrutinib (Berglöf *et al.*, 2015). Links between EGFR and the ephrin-receptor EPHA2 have already been reported (Larsen, Stockhausen and Poulsen, 2010). In addition, ADAM10 controls EGFR signalling, which in turn regulates EPHA1/2 signalling complexes in epithelial cells (Kaplan *et al.*, 2018). Coincidentally, ADAM10 protein expression is required for the activity of *S. aureus* α-hemolysin (Hla) on mammalian cells (Wilke and Wardenburg, 2010). Thus, the EPHA2 basal levels may be indirectly controlled by Hla during *S. aureus* infection of epithelial-like cells.

4.3.4. EPHA2 plays an important role on *S. aureus* cell infection

Internalization into the host cell is a crucial step to successfully establish infection for intracellular pathogens. Recently, the Ephrin type-A receptor 2 (EPHA2) has been highlighted as a potential common host receptor for the intracellular internalization of many different pathogens (Du Toit, 2018). The implication of this tyrosine kinase receptor in infection has already been demonstrated in different intracellular bacteria such as *M. tuberculosis* (Khounlotham *et al.*, 2009), *Chlamydia trachomatis* (Subbarayal *et al.*, 2015) and enteropathogenic *E. coli* (Scholz *et al.*, 2015). It is also involved in the entry of Epstein-Barr (Chen *et al.*, 2018) and Hepatitis C (Lupberger *et al.*, 2011) viruses into the host cell. Therefore, we speculated that the facultative intracellular pathogen *S. aureus* might also

exploit this host membrane receptor to facilitate its internalization into epithelial cells.

In accordance with our hypothesis, exposure of EphA2 knockout cell lines to *S. aureus* resulted in a reduction of intracellular bacteria, specially at early time points of infection. These results suggest that EPHA2 is important for the successful invasion or internalization of *S. aureus*. Additionally, the EPHA2 receptor is linked to many other downstream proteins that may also be involved in the intracellular survival or proliferation of *S. aureus*. For instance, EPHA2 receptor activates focal adhesion kinases (FAK) (Miao *et al.*, 2000), which has been previously related to the internalization of *S. aureus* (Agerer *et al.*, 2005). EphA2 is further associated with SHC, ERK and JUN proteins that were also inhibited under Ibrutinib treatment and play different roles in intracellular infections as afore mentioned. Therefore, the host-membrane receptor EPHA2 represents a very promising drug-target for intracellular MRSA anti-infectives.

4.4. CONCLUSION

We screened a total of 140 host-directed drugs against intracellular MRSA infection and we identified three host-kinase inhibitors – Ibrutinib, Dasatinib and Crizotinib – that showed a potential effect against intracellular MRSA. Among these three inhibitors, we characterized the role of Ibrutinib during intracellular *S. aureus* infection. In the presence of Ibrutinib, host cell viability was partially restored after MRSA infection whereas intracellular bacterial survival as well as *S. aureus* phagosomal escape was hampered. We also carried a phosphoproteomics analysis

in MRSA-infected cells in the presence or absence of Ibrutinib and we found twenty proteins whose phosphorylation was inhibited under Ibrutinib treatment. In particular, we identified EPHA2 as a potential target of Ibrutinib. Accordingly, intracellular MRSA survival was significantly reduced in EPHA2 knockout cells compared to the wild type.

Undoubtedly, a novel drug discovery approach to combat intracellular bacterial infections is emerging and is focused on identifying and targeting the host molecular and/or metabolic factors hijacked by intracellular pathogens (Zumla *et al.*, 2016; Bravo-Santano *et al.*, 2018). Ibrutinib has already been described as an effective host-directed therapy for *S. flexneri* and *L. donovani* (Dragoi, Talman and Agaisse, 2013; Varikuti *et al.*, 2018) and this study suggests that this inhibitor could also be repurposed to halt intracellular *S. aureus* when used in combination with conventional antibiotics such as gentamicin. This could be a very promising strategy to achieve patient decolonization of *S. aureus*.

5. Identification of novel targets for host-directed therapeutics against intracellular *Staphylococcus aureus*

5.1. BACKGROUND

In the previous two chapters, I identified novel host-directed therapeutics against intracellular *Staphylococcus aureus* by means of metabolomics and high-throughput drugs screening. Furthermore, I found new host-targets by elucidating the mechanism of action of a drug candidate by phosphoproteomics. In this chapter, we explored the host factors co-opted by *S. aureus* during cell infection with a functional genomics approach.

There are different strategies available to determine host factors hijacked by intracellular pathogens, combining genomics, computational biology, proteomics and transcriptomics (Schwegmann and Brombacher, 2008). These strategies could lead to the identification or development of new host-targeted treatments to combat intracellular infections.

In particular, loss-of-function phenotypic analysis, such as RNA interference (RNAi), has been employed to identify host genes or proteins that are necessary for the pathogen internalization, growth or replication within mammalian cells (Agaisse *et al.*, 2005; Philips, Rubin and Perrimon, 2005; Ng *et al.*, 2007; Brass *et al.*, 2008). For example, a genome-wide RNAi analysis was carried out in Drosophila cells infected with the cytosolic bacterium *Listeria monocytogenes*. In this study, they identified 358 host genes that were potentially involved in *Listeria* infection, covering a wide range of host cellular functions. Among the identified genes, many of them were involved in either vesicle trafficking, signal transduction or cytoskeleton rearrangement (Agaisse *et al.*, 2005). Another study identified the host-kinase AKT1 as an important host molecular factor during *M. tuberculosis* and *S.* Typhimurium infections by using an RNAi approach coupled to automated microscopy (Kuijl *et al.*, 2007). In addition, the involvement of the host Abl kinase in the internalization of *Pseudomonas aeruginosa* into Drosophila cells was also achieved by a RNAi screening (Pielage *et al.*, 2008).

The assessment of gene function using RNAi approaches relies on degradation of specific messenger RNAs (mRNAs) to silence gene expression. In mammalian cells, RNAi is usually mediated by microRNAs (miRNA) that are non-protein coding transcripts. shRNAs are miRNAs that are processed into shorter RNAs and contain a short-hairpin structure. These shRNAs are then further processed into short double-stranded pieces of RNA called short-interfering RNAs (siRNAs). The molecular mechanism of gene silencing comprises the binding of one strand of the siRNA duplex to a protein-coding mRNA transcript within the multi-protein RNA-Induced Silencing Complex (RISC). Consequently, this interaction triggers

cleavage of the protein-coding mRNA by a nuclease in the RISC complex, thereby destroying the mRNA and silencing the expression of the gene (Sims *et al.*, 2011).

In this study, a shRNA screening approach was employed to identify novel host-genes that could be involved in *S. aureus* cell infection. One of the main advantages of shRNA screenings, compared to siRNA approaches, is their ability to stably integrate into the genome of mammalian cells, allowing the generation of established knockdown cell lines and therefore, longer experimental time-courses (Coussens *et al.*, 2011; Sims *et al.*, 2011). Furthermore, the availability of commercial shRNA libraries constitutes a powerful technology that makes it possible to perform large-scale loss-of-function genetic screenings in mammalian cells (Root *et al.*, 2006; Sims *et al.*, 2011).

The aim of this chapter was to identify host-factors exploited by *S. aureus* for host cell invasion and intracellular proliferation by employing an unbiased shRNA-screening approach coupled to intracellular MRSA infection and Illumina sequencing-based deconvolution.

5.2. RESULTS

5.2.1. Identification of novel host-genes involved in *S. aureus* cell infection

The effect of silencing 16,000 human genes during *S. aureus* cell infection was tested by using the Mission® LentiPlex® Human Pooled shRNA Library (Sigma-Aldrich). The shRNA library comprises over 75,000 shRNA constructs that were

divided in 10 different sub-pools. Over 13,500 genes were targeted by at least 5 different shRNA constructs to ensure reproducibility (Fig. 5.1).

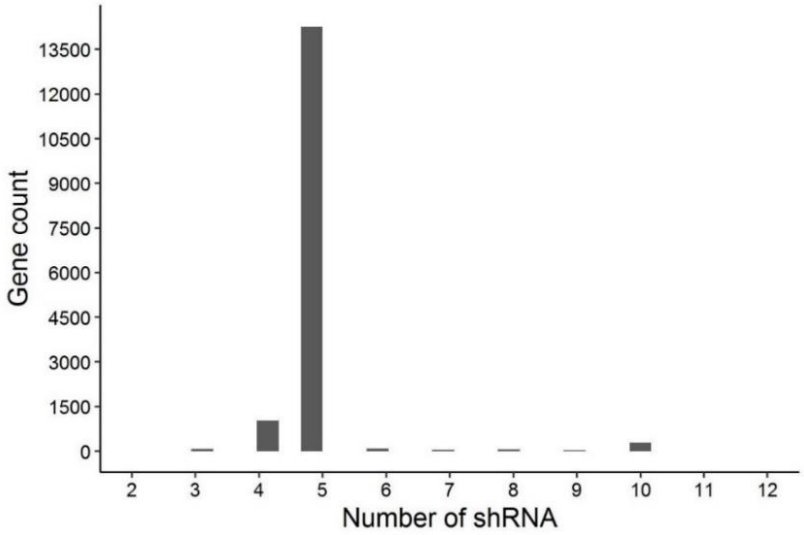

Figure 5.1. Distribution of shRNA constructs included in the Mission® LentiPlex® Human Pooled shRNA Library. Most of the genes that are included in the shRNA library are targeted by 5 different shRNA constructs.

I transduced HeLa cells with the shRNA library (Material and Methods 2.2.2) and performed puromycin selection to obtain cells stably expressing the shRNA library (shRNA-HeLa cells; Fig. 5.2.). Subsequently, we infected shRNA-HeLa cells with the clinically relevant *S. aureus* NCTC 13626 strain in the presence of vancomycin (Fig. 2.1) to investigate the effect of silencing specific genes on intracellular MRSA infection; uninfected shRNA-HeLa cells were used as negative control. After six hours of infection, we extracted total genomic DNA to determine by Illumina sequencing the total occurrence of each shRNA integrated on the shRNA-HeLa cells after infection (Fig. 5.2).

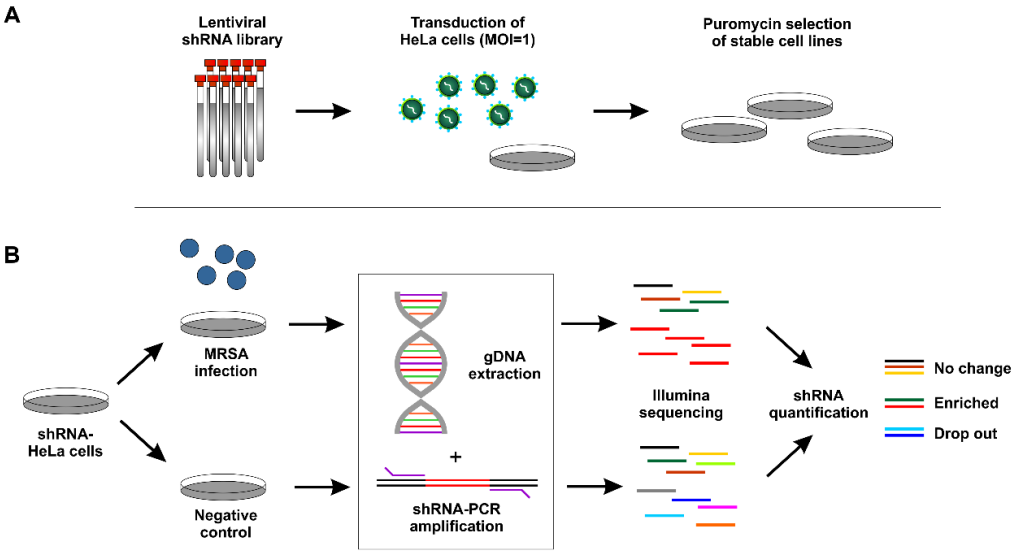

Figure 5.2. Layout of shRNA screening approach. (A) HeLa cells were first transduced with shRNA library until stable cells were produced (shRNA-HeLa). (B) shRNA-HeLa cells were infected with MRSA and genomic DNA was extracted to determine by Illumina sequencing the occurrence of each shRNA after infection.

By calculating the ratio between the occurrence of all shRNA constructs targeting the same gene found in *S. aureus*-infected cells (sample) versus the uninfected condition (control), we identified 5,674 shRNAs that were under-represented after *S. aureus* infection, whereas 9,997 shRNAs were over-represented when compared to uninfected cells (Fig. 5.3). Over-represented shRNA vectors may increase the host cell viability (and therefore their copy number) by depleting proteins used by the pathogen to enter or proliferate inside the host cells. Therefore, our downstream analysis was focused on genes targeted by over-represented shRNAs after *S. aureus* cell infection.

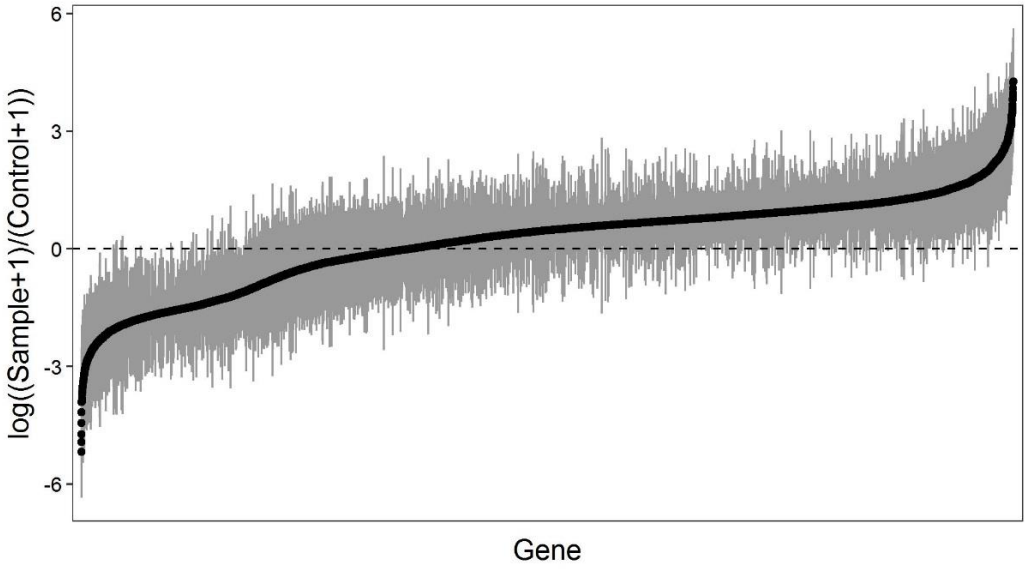

Figure 5.3. shRNA screening in HeLa cells after *S. aureus* infection. The relative quantity of each shRNA found in MRSA-infected cells was divided by the relative quantity found in uninfected cells and values were Log-transformed (Log+1). Mean and SE were calculated for those shRNAs that were targeting the same gene and plotted into the above graph. Means and SE are represented as black dots and grey lines, respectively. Black dots become as a continuous black line due to the high number of genes. This figure was provided by Pablo Capilla-Lasheras.

Results were firstly visualized in a volcano plot to identify hits based on their significance and magnitude of change, where 38 genes were highlighted after applying a very strict cut-off (log_2 fold change ≥3, $-log_{10}$ p-value ≥1.3) (Fig. 5.4).

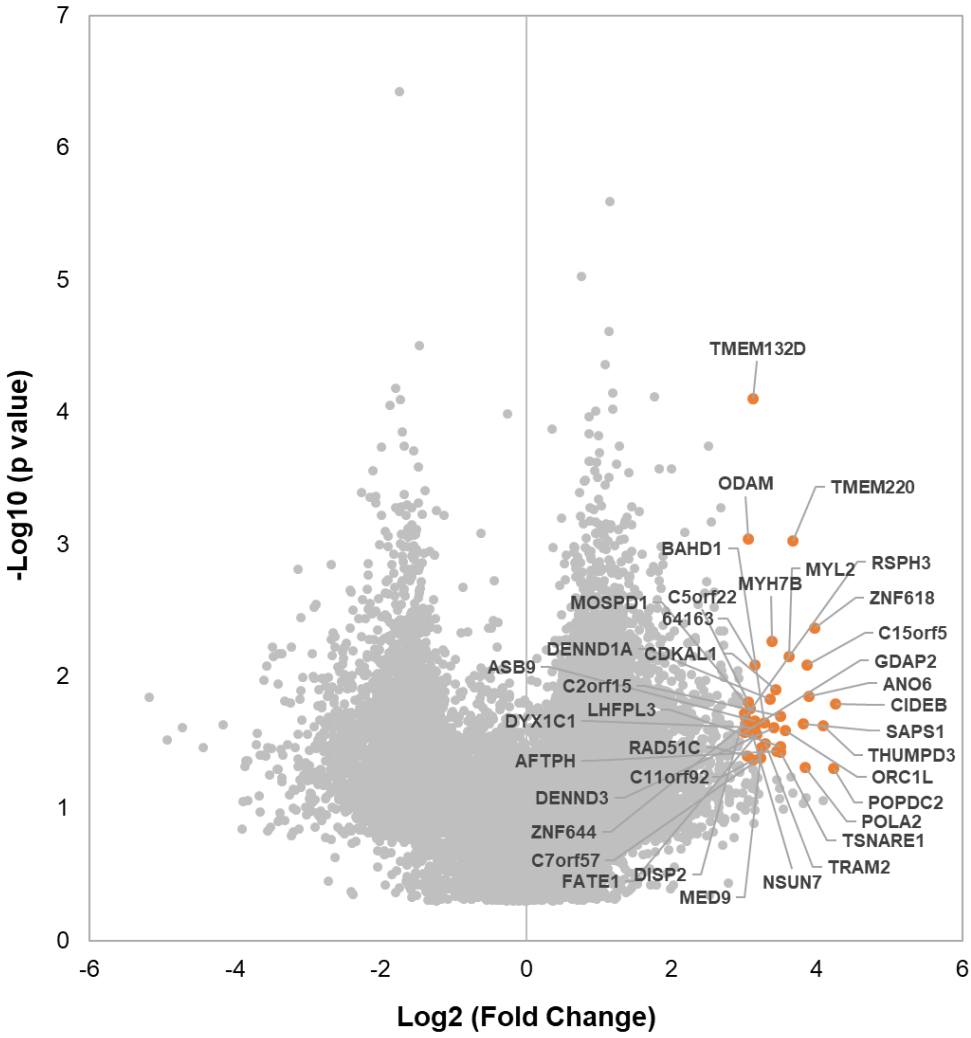

Figure 5.4. Volcano plots of the shRNA screen results. Screening results of 16,000 genes are ranked by fold-change and significance. Orange dots comprise genes with Log2 fold change of the mean ratio above 3 and -Log10 p-value higher than 1.3.

However, the silencing effect of empirically-designed shRNA vectors could be limited by their variable efficiency and specificity (Khandelwal *et al.*, 2015). Therefore, we further interrogated our screening results by considering the percentage of shRNAs that were under- or over-represented for each gene to estimate the consistency of the silencing effect. This is important because a consistent silencing effect produced by multiple shRNA constructs targeting the same gene is less likely to be due to an off-target effect (Root *et al.*, 2006; Sims *et al.*, 2011). We first established several cut-offs to identify the most interesting gene

candidates in our model of infection, which included a mean ratio greater than 1 (LogRatio >1) compared to uninfected cells, a standard error below 2 (SE <2) and a consistency over 60% (i.e. 60% of gene-specific shRNAs caused an effect in the same direction). By applying these criteria, we filtered 2,888 potential genes (Fig. 5.5).

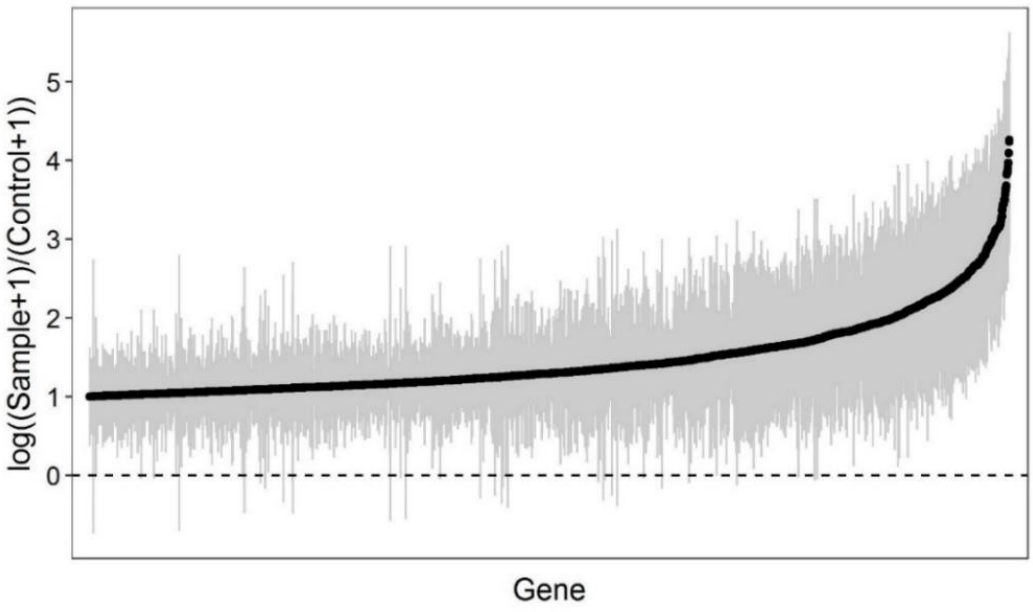

Figure 5.5. shRNA screening data after applying a strict cut-off. shRNA screening data were filtered by establishing three criteria: mean ratio higher than 1 (LogRatio >1) compared to the uninfected control, standard error lower than 2 (SE <2) and a consistency higher than 60%. This figure was provided by Pablo Capilla-Lasheras.

The identified genes were then functionally classified by using DAVID Bioinformatics Resources 6.8 (Huang, Sherman and Lempicki, 2009) and the genes were categorized in 63 potential cellular pathways (Fig. 5.6). The pathways with the highest number of genes were cytokine-cytokine receptor interaction and PI3K-Akt signalling pathways; both are implicated in a wide range of cellular functions, including cell growth, survival, differentiation and death. We also identified cellular pathways that may be important during *S. aureus* cell infection, e.g. regulation of autophagy, regulation of actin cytoskeleton, MAPK signalling and Toll-like

receptor signalling pathways. To investigate the possible function of those genes that are likely to be more biologically relevant for *S. aureus* cell infection, we used the GeneCards database (Stelzer *et al.*, 2011).

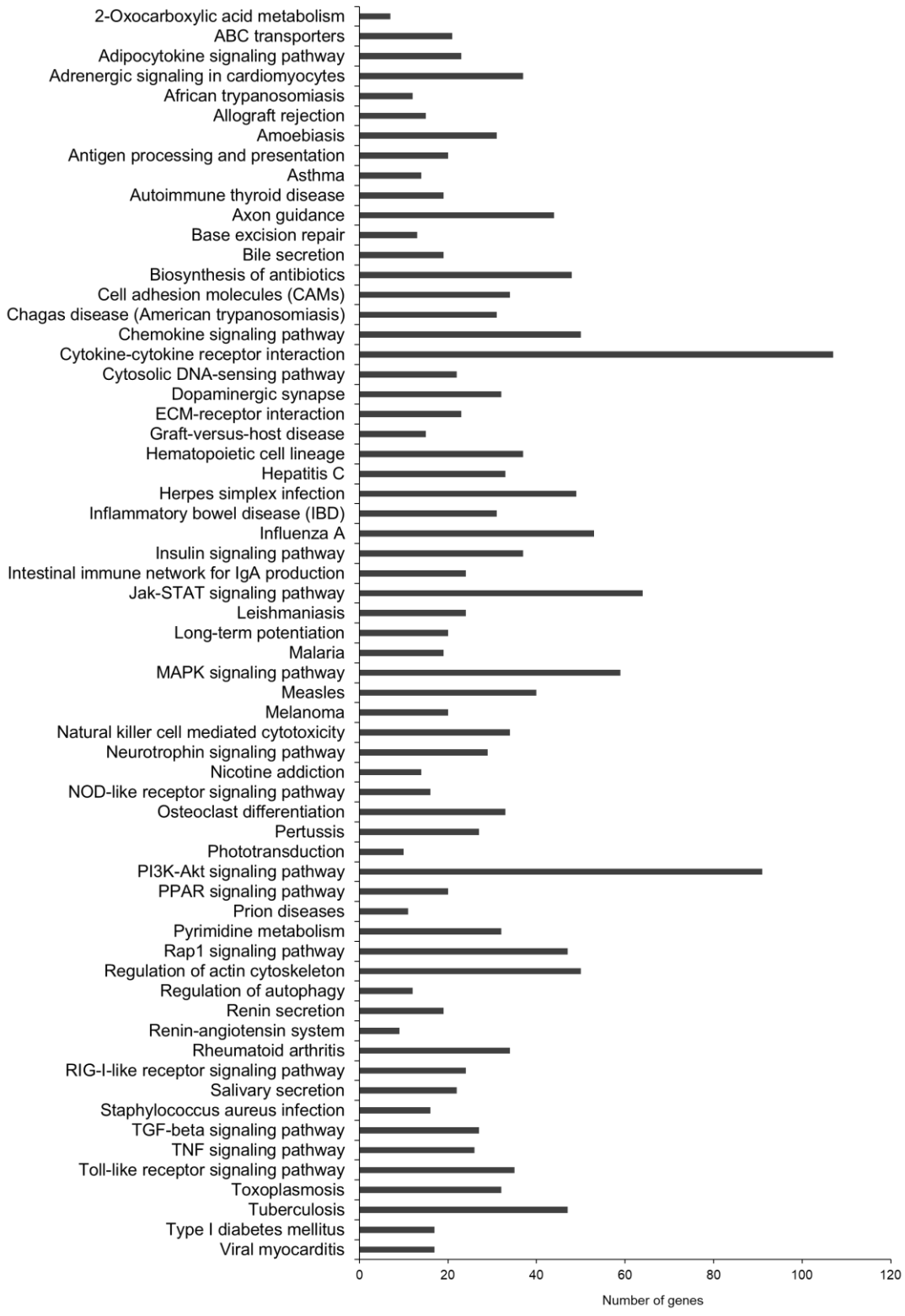

Figure 5.6. Functional classification of genes identified during the shRNA screen. KEGG pathways were analysed employing DAVID Bioinformatics Resources 6.8.

Following all the afore mentioned principles (i.e. magnitude and significance of change, consistent silencing effect and biological relevance), we selected a total of

29 genes for further validation (Fig. 5.7), of which seven – *CDKAL1*, *MYL2*, *NSUN7*, *ORC1L*, *POLA2*, *TRAM2* and *ZNF618* – were also identified in our previous Volcano plot analysis (Fig. 5.4).

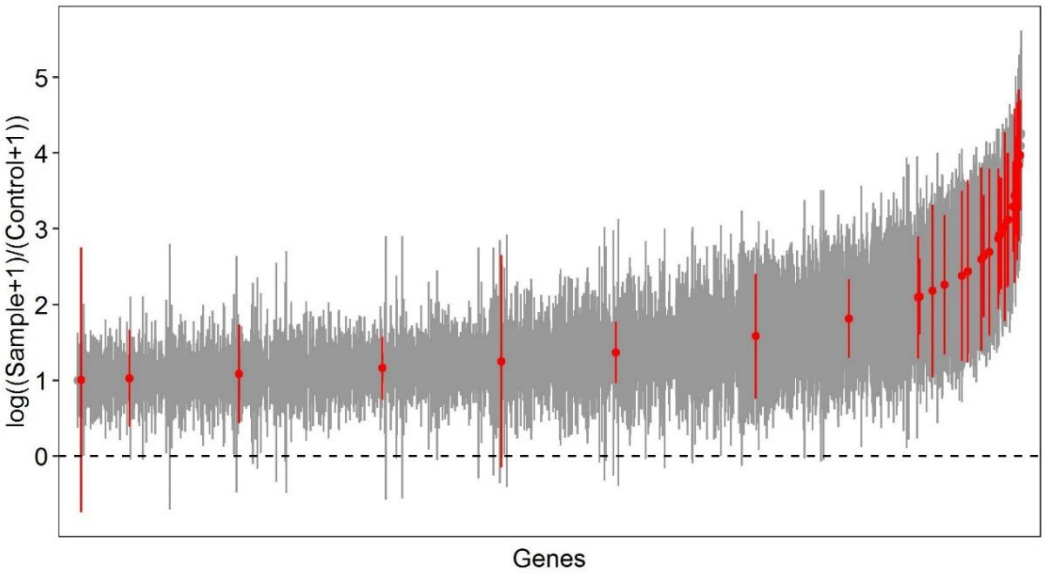

Figure 5.7. Twenty-nine genes were selected for further validation. High effect in the shRNA screening after MRSA infection and/or a biological relevance for intracellular pathogenesis comprise the two criteria to choose the most interesting hits. Selected genes are highlighted in red. This figure was provided by Pablo Capilla-Lasheras.

The twenty-nine selected-genes along with a summary of the information obtained from the shRNA screening are listed in table 5.1.

NCBI GeneID	Gene	Number shRNA	Positive hits	Negative hits	Consistency (%)	Mean ratio	SE ratio
83734	*ATG10*	5	4	1	80	1.002	1.748
57099	*AVEN*	5	3	0	60	1.083	0.649
10295	*BCKDK*	9	8	1	89	1.365	0.405
79780	*CCDC82*	5	4	0	80	3.030	1.248
922	*CD5L*	5	4	1	80	2.379	1.126
9308	*CD83*	5	4	1	80	1.250	1.404
54901	*CDKAL1*	5	5	0	100	3.438	1.149
1212	*CLTB*	5	3	1	60	1.581	0.823
1438	*CSF2RA*	5	4	0	80	2.259	0.921
54629	*FAM63B*	5	5	0	100	2.596	1.212
439996	*IFIT1B*	5	4	1	80	2.441	1.207
3775	*KCNK1*	5	5	0	100	2.869	0.930
1955	*MEGF9*	5	4	0	80	2.638	0.806
4633	*MYL2*	5	5	0	100	3.624	1.045
10398	*MYL9*	5	5	0	100	2.938	0.733
4753	*NELL2*	5	4	0	80	2.095	0.804
79730	*NSUN7*	5	5	0	100	3.111	0.883
4998	*ORC1L*	5	5	0	100	3.571	0.646
23649	*POLA2*	5	5	0	100	3.838	1.002
84440	*RAB11FIP4*	5	4	1	80	1.161	0.419
25782	*RAB3GAP2*	5	3	2	60	1.028	0.632
347517	*RAB41*	5	5	0	100	2.692	1.102
285641	*SLC36A3*	5	5	0	100	2.915	0.780
8501	*SLC43A1*	5	5	0	100	3.123	0.878
7097	*TLR2*	5	5	0	100	2.179	1.141
8794	*TNFRSF10C*	5	5	0	100	2.104	0.503
9697	*TRAM2*	5	5	0	100	3.292	0.601
127733	*UBXN10*	5	4	1	80	1.813	0.517
114991	*ZNF618*	5	5	0	100	3.970	0.732

Table 5.1. Twenty-nine genes were selected from the shRNA screening for further validation. Each gene is identified with its name along with the NCBI identification number. Positive hits, refers to the shRNAs constructs whose ratio was a positive number and thus, the representation of that shRNA construct was higher in the MRSA-infected sample than the uninfected control. By contrast, negative hits include shRNAs whose quantification in the MRSA-infected sample was lower than the uninfected sample. Consistency is the percentage of shRNAs that produce a similar silencing effect. Means and standard errors (SE) were calculated for the different shRNAs constructs that target the same gene.

By choosing these genes, we covered a diverse range of cellular pathways that may be exploited by MRSA during intracellular infection as well as some other novel genes whose functions have not been thoroughly described.

Among the shortlisted genes candidates, the Autophagy Related 10 (*ATG10*) gene is directly involved in cell autophagy (Jung *et al.*, 2010; Bernard *et al.*, 2015). Other genes are associated with programmed cell death or apoptosis, such as Apoptosis and Caspase Activation Inhibitor (*AVEN*) and CD5 molecule-like (*CD5L*) genes. *RAB* genes are linked to membrane trafficking (Hutagalung and Novick, 2011) and, indeed, several intracellular pathogens hijack host-RAB proteins during infection (Spanò and Galán, 2017). The components of the cell cytoskeleton also play an important role during intracellular infection, and we identified at least three proteins, CLTB and MYL2/9, which may be involved in cytoskeleton rearrangement (Fletcher and Mullins, 2010). We also observed proteins linked to the host cell metabolism, such as the branched-chain amino acids regulator (BCKDK) and the amino acids transporters SLC43A1 and SLC63A3, which could be important for intracellular MRSA survival (Bravo-Santano *et al.*, 2018). Additionally, we also included the CD83-molecule (*CD83*) and Toll-Like Receptor 2 (*TLR2*) genes in our downstream analysis, which are involved in a wide range of host immune responses to fight against bacterial and viral infections (Christiaansen, Varga and Spencer, 2015; Mukherjee, Karmakar and Babu, 2016).

To validate our results, we used individual shRNA constructs with the strongest silencing effect for each gene to produce individual knockdowns (Table 2.7). in HeLa cells for further investigation. An established cell line expressing an shRNA that does not silence any human gene (Non-target HeLa cells) was used as a negative control in all downstream experiments.

When silencing a gene that is essential for the successful intracellular infection of *S. aureus*, an increase in host cell viability and a reduction in intracellular bacterial load is expected. Hence, we first assessed host cell viability of the selected 29 knockdowns after MRSA infection. We performed infection assays with two different strains of MRSA – *S. aureus* NCTC 13626 (Healthcare-Acquired MRSA) and *S. aureus* USA300 (Community-Acquired MRSA; Fig. 5.8) – in the presence of vancomycin and gentamycin, respectively (Fig. 2.1)

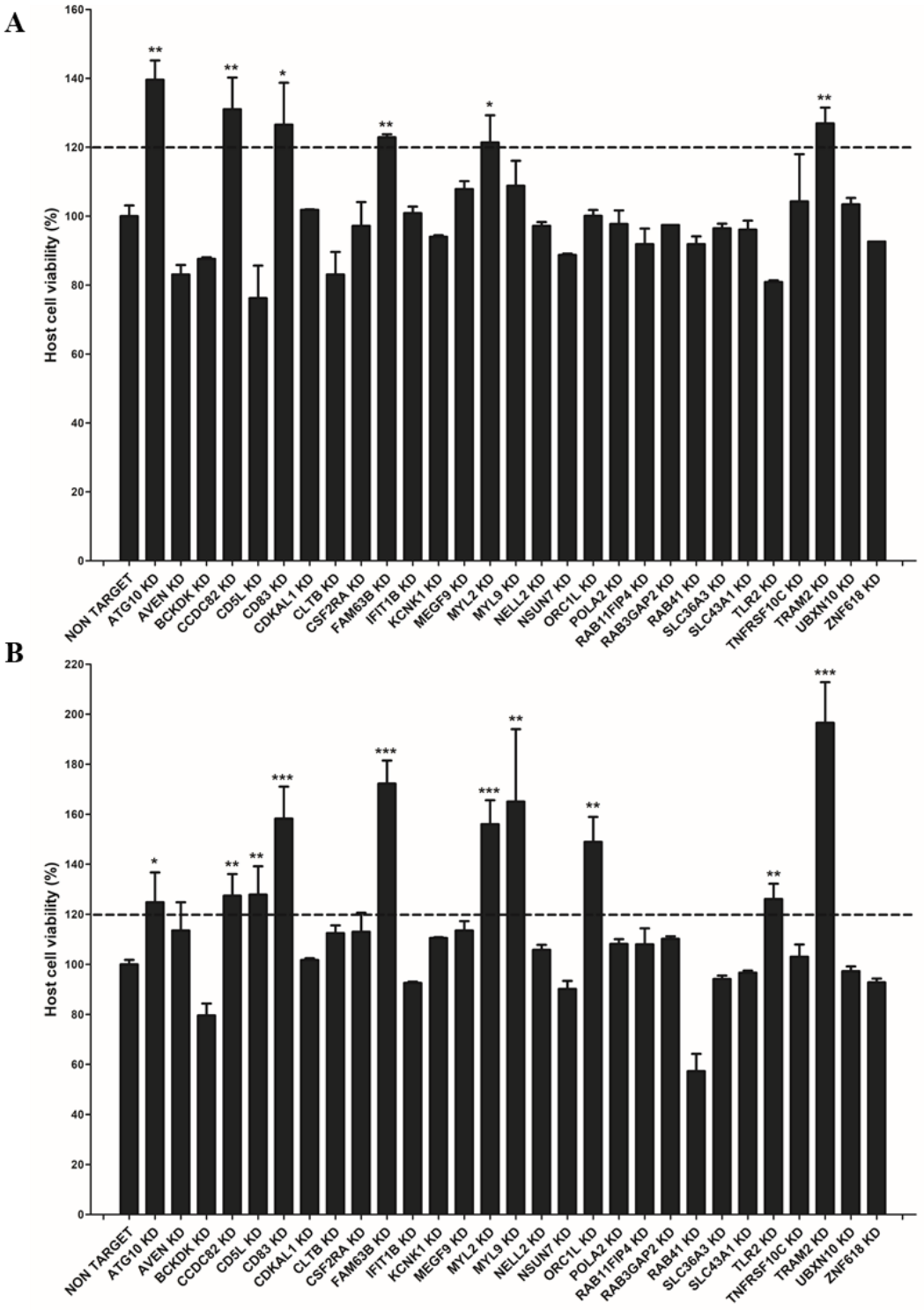

Figure 5.8. **Validation of the screening results.** Host cell viability after intracellular infection with *S. aureus* NCTC 13626 strain (A) and *S. aureus* USA300 LAC strain (B). After 6 hours of MRSA infection, cell viability was quantified by flow cytometry using a double staining of Annexin V-FITC and propidium iodide. Cell viability was normalized in relation to the non-target HeLa cells used as negative control. Data are expressed by means ± SE of two biological replicates performed in duplicates. Statistical differences were tested using Student´s t-test against non-target control. p-value ≤0.05 (*); ≤0.01 (**); ≤0.001 (***).

Overall, the effect on host cell viability of the different knockdowns was more noticeable after infection with *S. aureus* USA300 strain (Fig. 5.8B), where we found larger and more consistent differences among knockdowns compared to the non-target control. However, the effect of the gene silencing was generally very similar in both MRSA strains, with few exemptions. For example, *CD5L* and *TLR2* knockdowns showed opposite effects depending on the strain employed (Fig. 5.8). In addition, cell viability after silencing *ORC1L* was not affected after *S. aureus* NCTC 13626 infection, while it was significantly increased during *S. aureus* USA300 infection (Fig. 5.8).

These differences in host cell viability were probably strain-dependent and in order to recognize the most interesting hits, we selected knockdowns whose cell viability was at least 1.2-fold higher than non-target cells after *S. aureus* infection. Accordingly, silencing *ATG10, CD83, CCDC82, FAM63B, MYL2* or *TRAM2* genes resulted in a significant increase on host cell viability after *S. aureus* NCTC 13626 infection (Fig. 5.8A). In the case of *S. aureus* USA300 infection, the most significant effects were also observed with *ATG10, CD83, CCDC82, FAM63B, MYL2* and *TRAM2* knockdowns and we further identified some strains-specific hits including *CD5L, MYL9, ORC1L and TLR2* knockdowns (Fig. 5.8B).

In summary, we selected *CD83, FAM63B, MYL2* and *TRAM2* knockdowns for further analysis since the silencing of these four host genes resulted in increased host cell viability regardless of the strain that was employed, and these genes were not targeted before to halt *S. aureus* cell infection. In addition, ATG10 could be considered a good internal control, because it is well-known that autophagy is essential for *S. aureus* intracellular survival (Bravo-Santano *et al.*, 2018).

5.2.2. Silencing of host-genes *CD83*, *FAM63B*, *MYL2* and *TRAM2* halts intracellular *S. aureus* replication

We speculated that the increase in host cell viability after silencing a gene of interest may be caused by a reduction in the intracellular MRSA load within the host-cell. To test this hypothesis, we assessed the intracellular growth kinetics of *S. aureus* in *CD83, FAM63B, MYL2* and *TRAM2* knockdown cell lines (Fig. 5.9). Intracellular bacteria were quantified by colony forming units (CFU) counting after two and six hours post infection. The intracellular growth coefficient (IGC) was calculated by normalizing the number of bacteria recovered after six hours of infection by the initial time point (two hours).

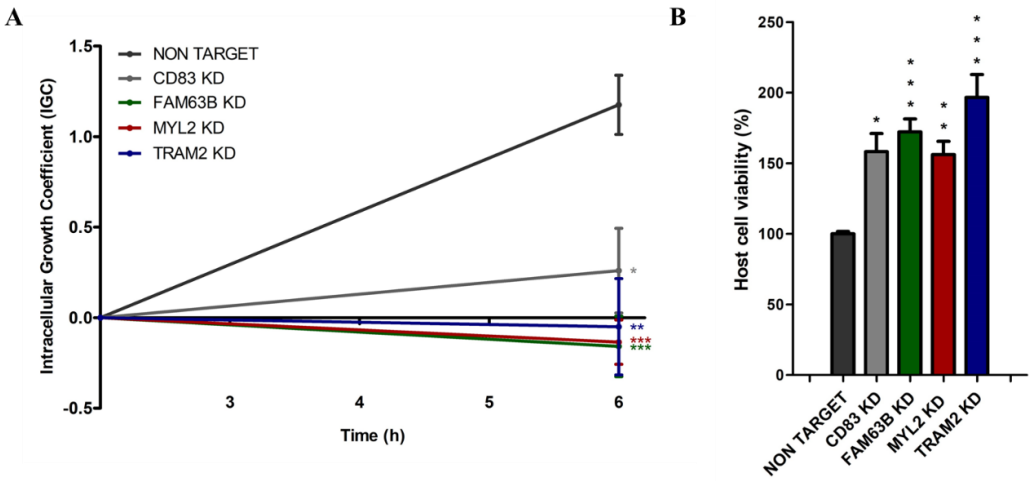

Figure 5.9. Bacterial and host cell viability after *S. aureus* infection in CD83, FAM63B, MYL2, and TRAM2 knockdowns. (A) Intracellular bacteria were quantified by CFU counting after 2 and 6 hours of infection. Number of bacteria recovered after 6 hours of infection was normalized by the initial time point (2 hours) to calculate the intracellular growth coefficient (IGC). (B) Cell viability was quantified in parallel after 6 hours of infection by flow cytometry, using a double staining of Annexin V-FITC and propidium iodide. Non-target HeLa cells were employed as negative control and cell viability was normalized in relation to the control's viability. Data are expressed by means ± SE of three experiments performed in duplicates. Statistical differences were tested using Student´s t-test against non-target control. p-value ≤0.05 (*); ≤0.01 (**); ≤0.001 (***).

A clear inhibitory effect on the intracellular growth was observed in every knockdown tested in comparison to the non-targeted control; with the most pronounced effects after silencing *FAM63B, MYL2* or *TRAM2* (Fig. 5.9A). Host cell viability was measured in parallel by flow cytometry (Fig. 5.9B), confirming that the number of viable cells in every knockdown was at least 50 % higher than the non-targeted control.

Taking into consideration both host cell viability and intracellular kinetics assays, two genes were consistently identified in all analyses: *MYL2* and *TRAM2*. However, since the host cell viability was restored more significantly when *TRAM2* was silenced compared to *MYL2*, this gene was chosen for further downstream experiments.

5.2.3. Characterization of the host gene *TRAM2* on *S. aureus* cell infection

I first confirmed the silencing of *TRAM2* gene in the knockdown measuring TRAM2 protein levels by Western-blot. Accordingly, levels of TRAM2 were reduced by 50% in the knockdown when compared to the non-targeted control (Fig. 5.10A/B).

I then evaluated the effect of *S. aureus* infection on the protein levels of TRAM2 in wild type HeLa cells at different time points (two and six hours post-infection) and multiplicities of infection (MOI 10 and 100) (Fig. 5.10C/D). As a control of bacterial load, we detected *S. aureus* Protein A by Western-blot, whose levels directly correlated with increasing times of infection and MOI (Fig. 5.10C).

Protein levels of TRAM2 changed in MRSA-infected HeLa cells at both time points, although some time-specific differences were observed (Fig. 5.10C). Protein levels of TRAM2 were increased after two hours of *S. aureus* infection to become up to 40% higher in infected HeLa cells (Fig. 5.10D). However, at six hours post-infection, I noticed a changed in the migration pattern of TRAM2 upon denaturing protein electrophoresis, indicating that it may have been subjected to post-translational modification such as proteolysis (Fig. 5.10C). This change in the migration pattern was proportional to the MOI employed for the infection, suggesting that *S. aureus* may be interfering with the post-translational modification of TRAM2 to promote its own survival or replication. This strategy is commonly employed by many bacterial pathogens to control host proteins during intracellular infection (Ribet and Cossart, 2010).

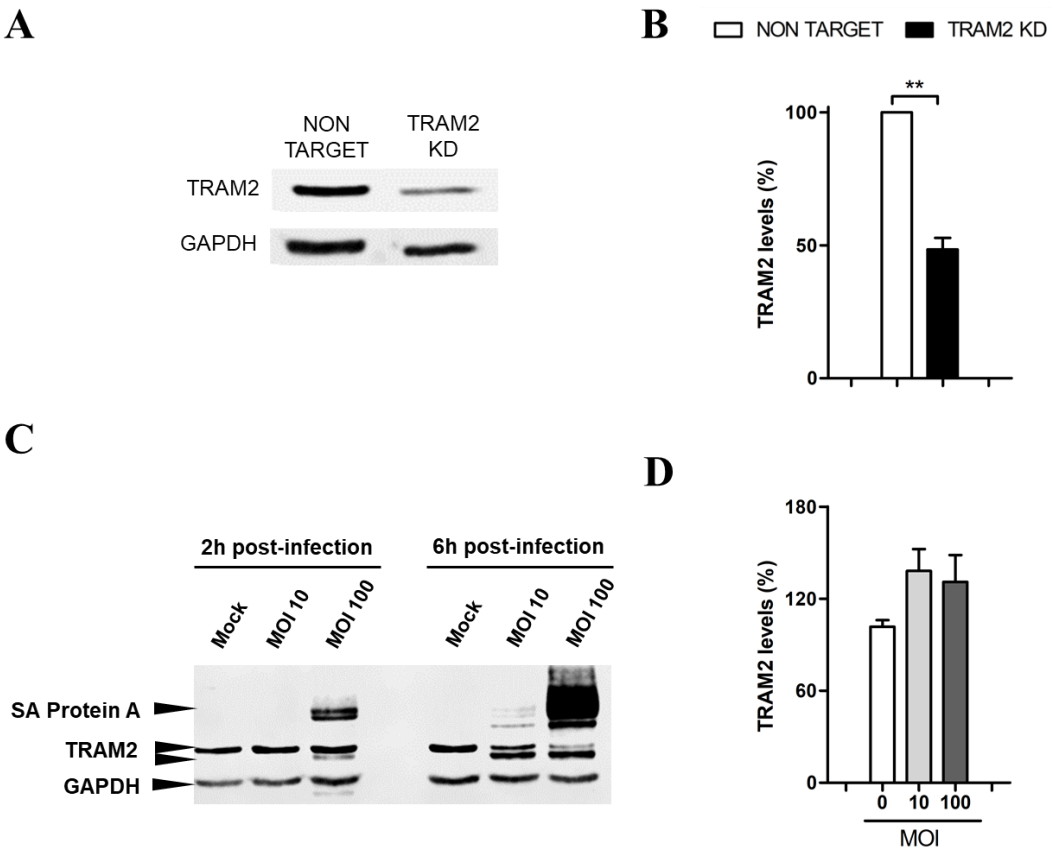

Figure 5.10. Validation of TRAM2 Knockdown in HeLa cells and TRAM2 protein levels in HeLa cells during *S. aureus* cell infection. (A) Cell extracts were analysed by Western-blot against TRAM2, and GAPDH was used as loading control. (B) Quantification of TRAM2 protein levels observed by Western-blot. (C and D) HeLa cells were infected with MRSA USA300 (MOI 10 and 100) and cell lysates were collected after 2 and 6 hours post-infection. (C) Cell extracts were analysed by Western-blot against TRAM2 and GAPDH antibody was employed as loading control. (D) Quantification TRAM2 protein levels observed by Western-blot at time point 2. Data are normalized to GAPDH levels and are expressed as means ± SE of three biological replicates. Student´s t-tests were performed to validate statistical significance across conditions. p-value ≤0.01 (**). Uninfected cells (Mock) were used as negative control.

Given that TRAM2 is a membrane protein, we hypothesized that it may be involved in the maturation of the vacuole containing *S. aureus*. Accordingly, TRAM2 silencing did not have an effect during early time points of infection (i.e. two hours post-infection), pointing towards a role on intracellular proliferation rather than cell entry (Fig. 5.11).

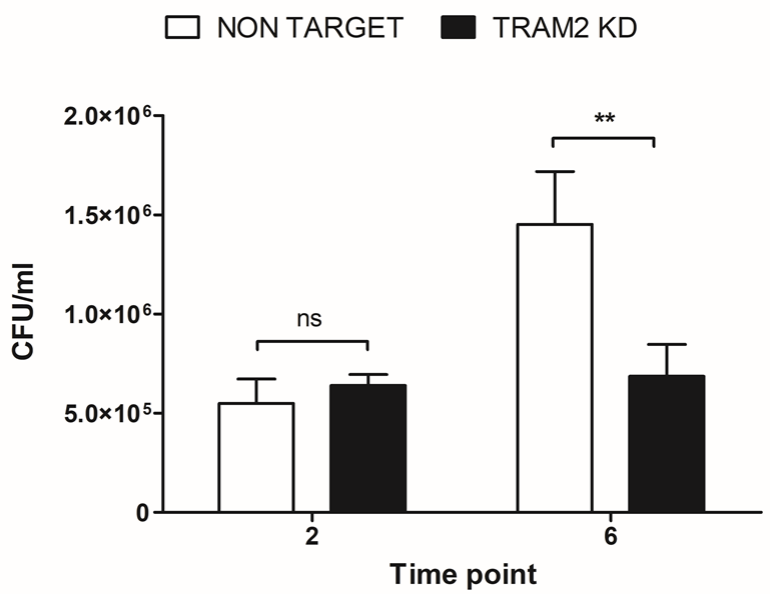

Figure 5.11. Intracellular MRSA infection is significantly hampered after 6 hours of infection. Non-target and TRAM2 KD HeLa cells were infected with MRSA (MOI 100) and CFU were counted after 2 and 6 hours of infection. Data are expressed as means ± SE of three biological replicates performed in duplicates. Student´s t-tests were performed to validate statistical significance across conditions; p-value ≤0.01 (**); (n.s) no statistical significance.

Therefore, I studied the subcellular location of *S. aureus* and TRAM2 during cell infection. HeLa cells stably expressing TRAM2-mCherry were infected with *S. aureus* USA300-GFP (Reichmann *et al.*, 2014). Co-localization assays were performed at 6 hours post-infection and MOI 100 by confocal microscopy, and the co-localization events between TRAM2-mCherry and intracellular *S. aureus* USA300-GFP were quantified (Fig. 5.12). In most of the cases, we observed large sacs of *S. aureus* cells surrounded by a scattered pattern of TRAM2-mCherry (Fig. 5.12A). In addition, some individual bacterial cells were enclosed by a membrane containing TRAM2-mCherry (Fig. 5.12B).

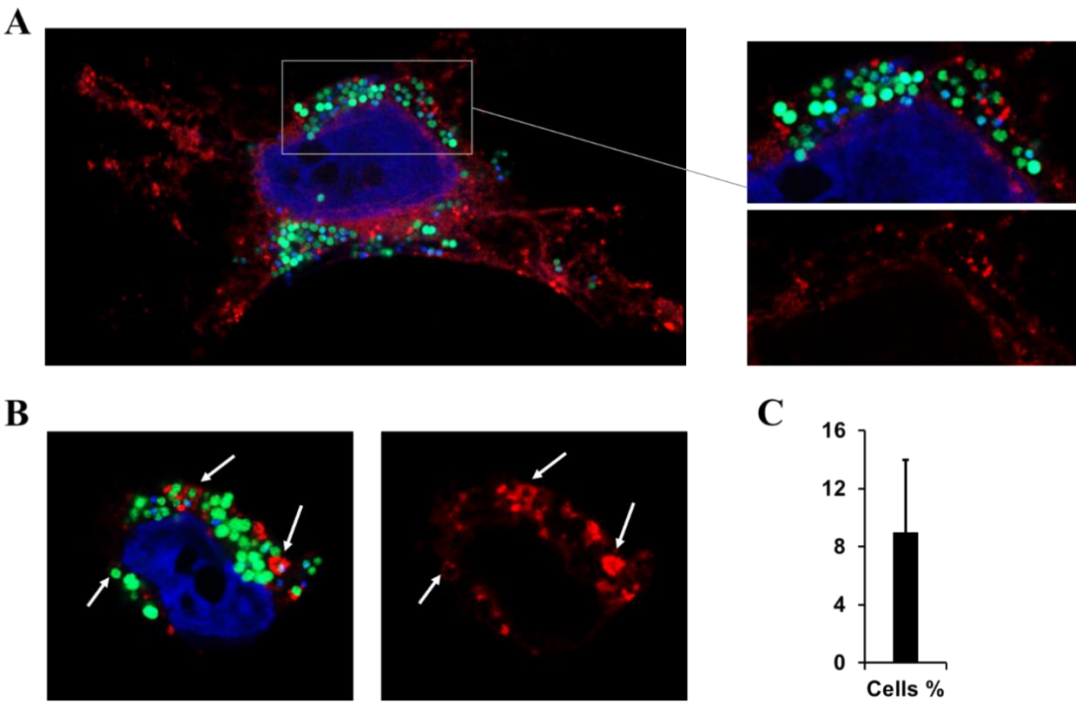

Figure 5.12. **Co-localization of MRSA-GFP and TRAM2-mCherry by confocal microscopy.** TRAM2-mCherry HeLa cells were infected with *S. aureus* USA300-GFP (MOI 100) and samples were collected after 6 hours of infection for confocal microscopy. DAPI staining was employed for nuclear staining. (A) Confocal picture showing a representative pattern of TRAM2-mCherry localization in *S. aureus* infected cells. (B) White arrows depict where co-localization between MRSA and TRAM2 was observed. (C) Percentage of infected cells showing co-localization of USA300-GFP and TRAM2-mCherry.

Nevertheless, these clear co-localization patterns of *S. aureus*-TRAM2 were restricted to approximately 9% of infected cells (Fig. 5.12C). Taken together the low number of localization events, we hypothesised that TRAM2 may have other roles during cell infection rather than the maturation of the vacuole containing intracellular *S. aureus*.

5.2.4. Thapsigargin impairs intracellular *S. aureus* infection

TRAM2 is part of the translocon, a complex of proteins involved in the transport of polypeptides across the endoplasmic reticulum (ER) membrane (Stefanovic *et al.*,

2004). The C-terminal end of TRAM2 interacts with the Ca^{2+} pump of the endoplasmic reticulum SERCA2b, which is necessary for the correct protein folding of proteins such as type I collagen (Stefanovic *et al.*, 2004). Based on this evidence, we hypothesized that by silencing *TRAM2* in HeLa cells we may in turn block Ca^{2+} pumps of the ER (e.g. SERCA2b), and consequently alter the maturation of proteins that are essential for *S. aureus* during cell infection. To test this hypothesis, I performed *S. aureus* infection assays in the presence of Thapsigargin, a selective SERCA inhibitor (Lytton, Westlin and Hanley, 1991), and we measured the host cell viability and intracellular MRSA survival.

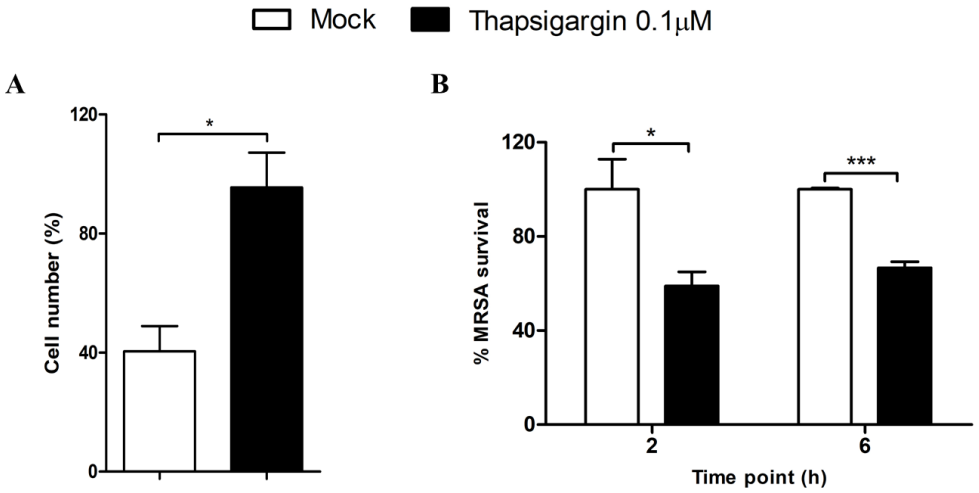

Figure 5.13. Thapsigargin treatment during *S. aureus* infection increases host cell viability whereas the intracellular bacterial load is reduced. HeLa cells expressing mCherry were infected with *S. aureus* USA300-GFP (MOI 100) for 2 and 6 hours in the presence of DMSO (Mock) or 0.1 μM of Thapsigargin. (A) Host cell viability was quantified after 6 hours of infection by flow cytometry. Cell viability was normalized by the percentage of uninfected and untreated cells. (B) Intracellular MRSA survival was quantified by measuring the percentage of host cells with intracellular *S. aureus* USA300-GFP after 2 and 6 hours of infection. Data are expressed as means ± SE of two different experiments performed in duplicates and Student´s t-tests were performed to validate statistical significance across conditions. p-value ≤0.05 (*); ≤0.001 (***).

We employed a low concentration of this inhibitor (0.1 μM), because higher doses have been reported to be cytotoxic (Stetler *et al.*, 2011; Sehgal *et al.*, 2017). In the presence of Thapsigargin, host cell viability was enhanced at 6 hours post-infection

(Fig. 5.13A). Moreover, intracellular MRSA survival was reduced by two-fold at early time points of infection (Fig. 5.13B). Conversely, we did not observe any significant differences in *S. aureus* growth curves in vitro in the presence of Thapsigargin (Fig. 5.14). Therefore, our findings suggest that a block of Ca2+ pumps of the ER mediated by Thapsigargin halts intracellular *S. aureus* survival in HeLa cells.

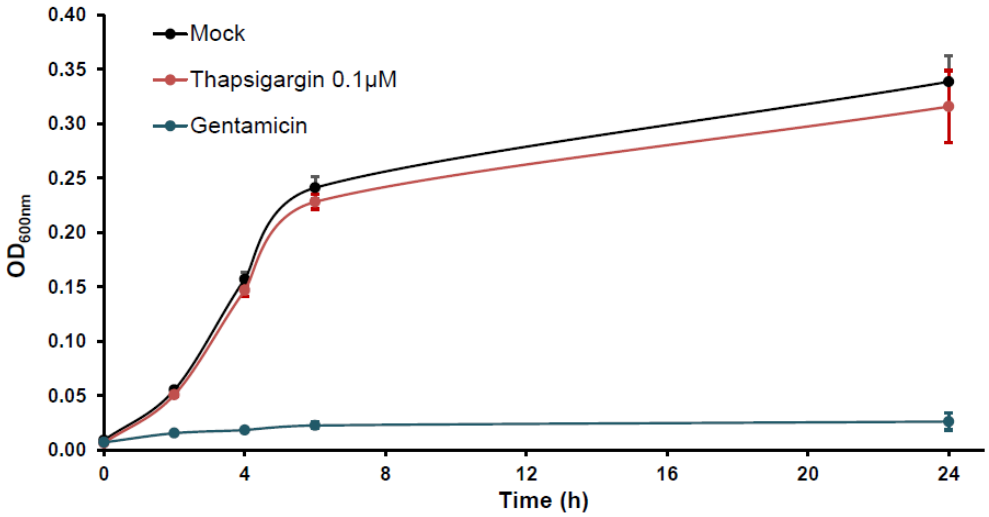

Figure 5.14. Thapsigargin does not directly affect *S. aureus* growth. *S. aureus* USA300 was grown in the presence of DMSO (Mock), Thapsigargin (0.1 µM) or gentamicin. Absorbance (OD_{600nm}) was measured at 2, 4, 6 and 24 hours to produce bacterial growth curves. Data are expressed as means ± SE of three different experiments performed in triplicates.

5.3. DISCUSSION

5.3.1. A functional genomics approach identifies novel host-genes essential for *S. aureus* during cell infection

The coupling of RNA interference (RNAi) with ultra-fast sequencing provides an exceptional opportunity to simultaneously screen the implication of thousands of

host-genes in different areas of research. In particular, RNAi approaches have been employed before to screen novel host-genes implicated in intracellular bacterial (Jayaswal *et al.*, 2010), parasitical (Moser, Pollard and Knoll, 2013) and viral (Hirsch, 2010) infections.

The main limitation of this high-throughput approach is the possibility of encountering phenotypes caused by off-target effects. To minimize this, transductions should be performed with a low multiplicity of infection (Sims *et al.*, 2011), and different shRNA constructs targeting the same gene should be included in the pooled libraries (Root *et al.*, 2006). Accordingly, we performed lentiviral transductions using an MOI of 1, and the majority of genes included in the commercial shRNA library were targeted by at least five shRNA constructs (Fig 5.1).

By employing ultra-fast sequencing, we were able to rapidly estimate the occurrence of shRNAs in *S. aureus*-infected cells in relation to the uninfected control. Over-represented shRNA constructs may be silencing human genes that are important for the intracellular bacterial survival and thus, any of these genes could have an essential role on *S. aureus* cell infection. We found approximately 10,000 human genes that were targeted by shRNAs over-represented in HeLa cells at six hours post-infection.

Further validation of our preliminary screening results was necessary and thus, the number of potential candidates was filtered. A combination of high consistency of the silencing effect, magnitude of the change and biological relevance was employed to select genes for further downstream analysis. As a result, 29 human genes were selected for further validation by producing individual knockdowns in HeLa cells and testing host cell viability after intracellular MRSA infection. Among

these genes, we included a varied range of cellular processes, such as autophagy, apoptosis, membrane trafficking and cytoskeleton rearrangement, amino-acids metabolism and host-immune responses. Many of these host cell pathways have previously been described to play a role in intracellular infection and therefore, we evaluated how host cell viability was affected during *S. aureus* infection after individually silencing these host-genes.

Of particular significance is ATG10, which is a vital component of the autophagy machinery. ATG10 is essential for the formation of autophagosomes by mediating ATG12-ATG5 conjugation and the conversion of soluble LC3 into its membrane-bound form (Nemoto *et al.*, 2003). ATG10 silencing significantly restored cell viability after infection with two different MRSA strains (Fig. 5.4). Accordingly, intracellular *S. aureus* infection in non-phagocytic cells is halted with autophagy inhibitors such as dorsomorphin (Bravo-Santano *et al.*, 2018), and 3-Methyladenine treatment protects mice from MRSA pneumonia (Zhu *et al.*, 2018).

To further identify other host-targeted therapeutics against intracellular MRSA, we interrogated our shRNA screening results to identify four genes, i.e. *CD83*, *FAM63B*, *MYL2* and *TRAM2*, whose silencing in HeLa cells resulted in a significant reduction of intracellular *S. aureus* survival while host cell viability was enhanced after cell infection.

The protein encoded by *CD83* gene is a host membrane protein that is part of the immunoglobulin family and it is mainly expressed in mature dendritic cells (DCs). The main function of these antigen-presenting cells is the activation of host immune responses against invading pathogens (Schmidt, Nino-Castro and Schultze, 2012). Eukaryotic cells have evolved complex immune mechanisms to combat invading pathogens. Consequently, and in order to successfully establish infections,

intracellular microorganisms have also developed sophisticated mechanisms to subvert and overcome host immune responses (Reddick and Alto, 2014). Specifically, it has been shown that interleukin 10 (IL-10) – a cytokine produced by macrophages, T-cells, B-cells, mast cells and keratinocytes, among others – can be an effective immunosuppressive factor and therefore, the exploitation of this cytokine is a common mechanism to evade host immune responses by several pathogens. Some viruses induce the production of host IL-10, while others produce their own IL-10-homologs. Bacterial intracellular pathogens, such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*, are able to replicate within macrophages by inducing IL-10 (Redpath, Ghazal and Gascoigne, 2001). However, further investigation is needed to explain the molecular mechanism behind *CD83-S. aureus* interaction in cells that are not part of the immune system.

Although its function is unknown, FAM63B has been recently identified as an interaction partner of kinesin light chain-1 (KLC1), which is involved in the intracellular trafficking of vaccinia virus (Dodding *et al.*, 2011). Kinesins, dyneins and myosins comprise three classes of molecular motors that are part of the host cell cytoskeleton and are involved in many biological processes related to cell movement. Intracellular bacterial pathogens and viruses commonly use this host machinery to reach their intracellular niche, as well as to control the membrane remodelling of their containing vacuoles (Henry, Gorvel and Méresse, 2006). In particular, Kinesin-1 has been implicated in the intracellular dissemination of adenovirus and Herpes virus (Greber and Way, 2006; Dodding *et al.*, 2011; Zhou *et al.*, 2018). Moreover, *Salmonella* effector protein SifA targets the host protein SKIP, which down-regulates the recruitment of Kinesin-1 to the *Salmonella*-containing vacuole and thus, controls its dynamics (Boucrot *et al.*, 2005).

Therefore, FAM63B may play a role in the intracellular trafficking of *S. aureus* by targeting kinesin proteins.

Myosins comprise a superfamily of motor proteins found in actin filaments and have an essential role in the organization of actin cytoskeleton. Polymerization and depolymerization of actin filaments promote changes in cells shape and, along with myosin proteins, control the intracellular organization (Fletcher and Mullins, 2010). The host cell cytoskeleton is commonly hijacked by intracellular pathogens to support their own intracellular invasion, survival and replication and actin has been specifically described as a common target of many bacterial pathogens (Choe and Welch, 2016). As above mentioned, bacterial pathogens exploit host-actin for different purposes such as intracellular invasion, actin-based motility through cell cytoplasm and pathogen dissemination (Haglund and Welch, 2011). Likewise, the use of microtubules, motor proteins and intermediate filaments in intracellular invasion and dissemination has been highlighted (Haglund and Welch, 2011). For instance, the actin-base motor protein myosin 2 has been associated with the integrity and location of *Salmonella*-containing vacuoles (Wasylnka *et al.*, 2008). In addition, myosin 2 has been associated with the promotion of vesicle fission from the Golgi complex (Miserey-Lenkei *et al.*, 2010). Furthermore, there is a proportion of cytosolic *Shigella* that become covered with septin filaments in a myosin 2-dependent manner (Mostowy *et al.*, 2010). Moreover, the release of *Chlamydia*-intrusions into the extracellular space is mediated by actin, N-WASP, myosin 2 and GTPase Rho proteins (Hybiske and Stephens, 2007). Collectively, these studies suggest that the intracellular fate of many intracellular pathogens is determined by an organized collaboration between actin, microtubules, intermediate filaments and motor proteins. Therefore, the gene coding for the myosin light chains 2 (MYL2)

could be involved in the intracellular dissemination of *S. aureus* by controlling host actin cytoskeleton.

5.3.1.1. TRAM2 plays an important role during intracellular MRSA infection

Our findings showed that the human gene *TRAM2* is important for intracellular *S. aureus* infection. TRAM2 (Translocating chain-associated membrane protein 2) was initially identified as being responsible for the translocation of proteins across the endoplasmic reticulum membrane (Walter, 1992). Furthermore, TRAM2 interacts with the endoplasmic reticulum Ca^{2+} ATPase transporter SERCA2b, being involved in collagen type I protein folding (Stefanovic *et al.*, 2004). In addition, SERCA transporters pump cytosolic Ca^{2+} to the endoplasmic reticulum to maintain a constant Ca^{2+} gradient, which controls other key cellular functions such as cell growth, proliferation, differentiation and death (Berridge, Bootman and Roderick, 2003; Vandecaetsbeek *et al.*, 2009).

Different intracellular pathogens rely on the endoplasmic reticulum and its components to ensure intracellular survival and proliferation (Celli and Tsolis, 2015). For instance, *L. pneumophila*, an intravacuolar pathogen, hijacks host membrane components to contribute to the formation of *Legionella*-containing vacuoles that are mainly derived from endoplasmic reticulum material (Tilney *et al.*, 2001; Isberg, O'Connor and Heidtman, 2009). Similarly, *B. abortus* (Pizarro-cerda *et al.*, 1998; Celli *et al.*, 2003) and *C. trachomatis* (Derré, Swiss and Agaisse, 2011; Dumoux *et al.*, 2012) also exploit host-endoplasmic reticulum to promote their intracellular survival and replication within mammalian cells. In addition,

SERCA2b has been closely associated with Chlamydial infections in HeLa cells (Majeed *et al.*, 1999). In concordance with these observations, co-localization assays showed that several chlamydial antigens, such as major outer membrane protein (MOMP), inclusion membrane protein and lipopolysaccharides (LPS), were specifically associated with the ER and ER-markers (Giles and Wyrick, 2008).

Because of the interaction of TRAM2 and SERCA transporters, we investigated the intracellular *S. aureus* survival under treatment with Thapsigargin – a promising anticancer drug that is a specific host-SERCA inhibitor (Lytton, Westlin and Hanley, 1991; Mahalingam *et al.*, 2016). High doses of Thapsigargin lead to host cell death due to the ER stress caused by this inhibitor (Sehgal *et al.*, 2017). However, treatment of HeLa cells with low doses of Thapsigargin resulted in a notable reduction of intracellular *S. aureus*, similarly to the effect that we previously observed in TRAM2 knockdown cell lines. Furthermore, intracellular bacterial load was significantly attenuated at both time points of infection tested with Thapsigargin (at two and six hours post infection), whereas this reduction was only observed after six hours of infection in TRAM2 knockdown cells. Therefore, Thapsigargin may have multiple effects on *S. aureus* intracellular survival apart from the inhibition of Ca^{2+} pumps in the endoplasmic reticulum. Nevertheless, our findings suggest that the anti-cancer drug Thapsigargin may be repurposed to block *S. aureus* cell infection when combined with conventional antibiotics.

5.4. CONCLUSION

By using a functional genomics approach, we screened the role of 16,000 host-genes on intracellular *S. aureus* infection, to identify potential host targets that could be hijacked by *S. aureus* during intracellular infection. Here, we studied the human gene *TRAM2*, which plays an important role during *S. aureus* infection. TRAM2 is a transmembrane protein which is associated in the endoplasmic reticulum with SERCA Ca^{2+} pumps. Accordingly, a treatment of infected cells with Thapsigargin – a potent SERCA inhibitor – resulted in a significant reduction of intracellular *S. aureus*. Therefore, this study provides new insights into host-*S. aureus* interactions and a novel host-directed anti-infective strategy to halt *S. aureus* cell infection. Furthermore, data presented in this study serve as proof-of-principle of the functional genomics approach to unravel host molecular mechanisms hijacked by *S. aureus* during cell invasion and intracellular proliferation in human cells.

6. Conclusion

6.1. CONCLUDING REMARKS

A better understanding of host-*S. aureus* interactions during intracellular infection may lead to novel therapeutic strategies by targeting those host cellular pathways exploited by this versatile pathogen. In pursuing of this aim, this thesis unravelled some of the host metabolic pathways and molecular factors hijacked by *S. aureus* during intracellular infection as well as host-directed drugs that may be used to hamper intracellular *S. aureus* survival or proliferation.

The replication of an intracellular pathogen – including *S. aureus* – inside the host cell leads to numerous metabolic changes in the host cell as an adaptation of the pathogen to its new environment. In order to identify the specific host-metabolic pathways exploited by intracellular *S. aureus* to survive within mammalian cells, I characterized the host metabolic changes upon intracellular infection. Among all the metabolic changes observed in MRSA-infected cells, the low levels of glucose and amino acids along with an activated glutaminolysis pointed towards a starvation-state, which has previously been linked to autophagy. Autophagy was also activated in MRSA-infected cells as well as the host AMPK pathway, which is a key sensor of cellular energy homeostasis. The induction of autophagy by *S.*

aureus was probably due to its metabolic requirements within its intracellular environment and this hypothesis was tested by using Dorsomorphin, an inhibitor of the host-AMPK pathway. Treatment with dorsomorphin in HeLa cells reduced autophagy levels and intracellular MRSA load. Such effect of dorsomorphin against intracellular MRSA was further confirmed in a primary human cell line, HUVEC.

The identification of dorsomorphin – a host-directed drug, which achieved a reduction of intracellular MRSA in mammalian cells – encouraged us to look for other drugs that could have a similar effect against this pathogen. We therefore tested the effect of host-directed drugs, that were commercially available and already approved for other clinical purposes, on intracellular *S. aureus* infection. In order to screen dozens of drugs at the same time, we developed a high-throughput screening approach, which allowed us to easily quantify both viable host cells and intracellular bacteria. In this study, we screened a total of 140 host-directed drugs and found three host kinase inhibitors – Ibrutinib, Dasatinib and Crizotinib – whose treatment in HeLa cells significantly reduced intracellular MRSA. We further characterized the role of Ibrutinib on MRSA infection and carried out a phosphoproteomics analysis to reveal its mechanism of action. Although the phosphorylation of several proteins was inhibited by Ibrutinib treatment in HeLa cells, we particularly identified the host receptor EPHA2 as one of the potential targets of Ibrutinib in epithelial cells during MRSA infection. In line with this hypothesis, intracellular MRSA survival at early time points of infection was significantly hampered in EPHA2 knockout cells, suggesting that this host receptor may be involved in the invasion or internalization of *S. aureus*.

Moreover, we performed and validated another high throughput based on functional genomics to identify novel host-targets exploited by MRSA to support its

intracellular survival and/or replication. Using this approach, we found four potential candidates: *CD83*, *FAM63B*, *MYL2* and *TRAM2* and we specifically investigated the role of the host gene *TRAM2* on intracellular MRSA infection. The reduction of intracellular MRSA after six hours of infection suggested that TRAM2 protein is likely required for intracellular proliferation rather than for early steps of infection, such as invasion or internalization. In addition, TRAM2 has been associated with the endoplasmic reticulum SERCA Ca^{2+} pumps for the synthesis of collagen and hence, we investigated whether the SERCA inhibitor Thapsigargin had a similar effect on intracellular MRSA infection. Accordingly, intracellular MRSA proliferation was halted by the treatment with Thapsigargin in HeLa cells.

Overall, this study provides novel insights into host and *S. aureus* interactions during intracellular infection as well as new host-directed drugs that halt intracellular *S. aureus* growth. Furthermore, we have standardized and validated two high-throughput methods that could be applied to other intracellular pathogens (not only *S. aureus*) to identify novel host targets as well as host-directed drugs to improve the treatment of infections caused by intracellular bacteria.

Although we did not achieve complete clearance of intracellular MRSA, the combination of conventional therapies and host-directed drugs may improve the outcome of the infection. Therefore, targeting the host cellular pathways exploited by the facultative intracellular pathogen *S. aureus* could represent a promising therapeutic strategy. Table 6.1 highlights the host factors and pathways that have been described to be important for intracellular *S. aureus* infection so far, including the main findings of this thesis.

Host-factor	Putative function	Reference	Host-directed drug	Type
Adherence and Internalization				
α5β1-integrins	Internalization into non-phagocytic cells	Sinha et al. 1999	Volociximab *	Antibody
FAK	Internalization into non-phagocytic cells	Agerer 2005; Richter et al. 2016	PF-562271	Inhibitor
Src-mediated cortactin	Internalization into non-phagocytic cells	Agerer 2005; Richter et al. 2016	PP2	Inhibitor
β_1-integrins	Internalization into mast cells	Goldmann et al. 2015	R1295 *	Antagonist
Annexin 2	Internalization into epithelial cells	Ashraf et al. 2017		
(PI3K)-Akt	Internalization into endothelial cells	Oviedo-Boyso et al. 2011	Nelfinavir *	Inhibitor
αVβ3-integrin	Internalization into endothelial cells	McDonnell et al. 2016	Cilengitide *	Inhibitor
ERK	Internalization into osteoblast and Hep-2 cells	Ellington et al. 2001	SCH772984 *	Inhibitor
ERK1/2/MEK	Penetration into airway epithelial cells	Soong et al. 2011	UO126	Inhibitor
ERK	Invasion to fibroblasts	Zhao et al. 2017	PD98059	Inhibitor
Hsp60	Internalization into epithelial cells	Dziewanowska et al. 2000		
Hsc70	Internalization into 293T cells	Hirschhausen et al. 2010		
Desmoglein 1	Adherence to keratinocytes	Askarian et al. 2016		
Scavenger protein gp340	Internalization into A549 cells	Yang et al. 2014		
EGFR	Penetration into airway epithelial cells	Soong et al. 2011	BPDQ	Inhibitor
ROCK	Penetration into airway epithelial cells	Soong et al. 2011	Y-27632	Inhibitor
JNK	Penetration into airway epithelial cells	Soong et al. 2011	SP600125	Inhibitor
p38/MAPK	Penetration into airway epithelial cells	Soong et al. 2011	SB202190	Inhibitor
EPHA2	Invasion/Internalization into epithelial cells	This study	Ibrutinib	Inhibitor
CDK	Adhesion to human bronchial epithelial cells	Richter et al. 2016	Roscovitine	
PKA	Internalization into Thp1 macrophages	Miller et al. 2011; Richter et al. 2016	H-89	Inhibitor
PKC	Internalization into Thp1 macrophages	Miller et al. 2011; Richter et al. 2016	Bisindolylmaleimide-I	Inhibitor

Host-factor	Putative function	Reference	Host-directed drug	Type
Intracellular survival and proliferation				
ADAM10	Cleavage of adherens junction protein E-cadherin	Inoshima et al. 2011	GI 254023X	Inhibitor
AMPK	Induction of autophagy	This study	Dorsomorphin	Inhibitor
ERK	Induction of autophagy	This study	SCH772984 *	Inhibitor
TRAM2	Ca2+ pump to promote collagen synthesis	This study	Thapsigargin	Inhibitor
p38/MAPK	Subversion of autophagy	Neumann et al. 2016	Skepinone-L *	Inhibitor
PAK	Cytoeskeleton rearrangements	Richter et al. 2016	FRAX597 *	Inhibitor
MYL2	Cytoeskeleton rearrangements	This study	Blebbistatin *	Inhibitor
FAM63B	Intracellular trafficking	This study		
Actin	Promote bacterial movements within the host cell	Sinha et al. 1999; Schröder et al. 2006	Cytochalasin D	Inhibitor
Rab5	Promote bacterial movements within the host cell	Schröder et al. 2006		
NWASP	Production of actin-comet tails to facilitate movement	Schröder et al. 2006	Wiskostatin	Inhibitor
TMEM59	Activation of selective-autophagy	Boada-Romero et al. 2013		
RAPGEF3	Induction of autophagy	Mestre & Colombo 2012	Salirasib *	Inhibitor
RAP2B	Induction of autophagy	Mestre & Colombo 2012		
***S. aureus*-induced host cell death**				
Caspase 2	*S. aureus*-induced apoptosis	Imre et al. 2012	Z-VDVAD-FMK	Inhibitor
Caspase 9	*S. aureus*-induced apoptosis	Rudel et al. 2010	Z-LEHD-FMK	Inhibitor
NLRP3	*S. aureus*-induced pyronecrosis	Muñoz-Planillo et al. 2009	MCC950 *	Inhibitor

Table 6.1. Host molecular factors hijacked by *S. aureus* during intracellular infection and potential host-directed drugs against intracellular *S. aureus.* (*) Drugs that could potentially halt intracellular *S. aureus* by targeting the described host-pathway, but its effect has not been yet investigated.

6.2. LIMITATIONS AND FUTURE WORK

We have validated some of the host cellular pathways and factors – AMPK, EPHA2 and TRAM2 – that I identified as relevant for the intracellular survival of *S. aureus*. Nonetheless, there are many other unsolved questions that could be interesting to address in the future.

The experimental approaches we employed in this study – metabolomics, drug-screening, phosphoproteomics and shRNA screening – provided us with a long list of potential candidates that may be worthwhile to further investigate:

(i) Besides the starvation-status and upregulation of the host-AMPK pathway, another metabolic hallmark of MRSA-infected cells was the large increase of lower glycolysis metabolites (PEP and 3-PG). I hypothesize that such accumulation could be due to a downstream block caused by the inhibition of different enzymes such as pyruvate kinase or pyruvate dehydrogenase. Testing the effect of inhibitors of these two enzymes during MRSA infection could explain the increase of these two metabolites in MRSA-infected cells compared to the uninfected control.

(ii) From the host-directed drug screening, I characterized the role of Ibrutinib since it showed the highest effect, however, we also identified other tyrosine kinase inhibitors that could have a potential against intracellular *S. aureus*. One example is Imatinib, a BCR-ABL tyrosine kinase inhibitor, which is currently under clinical trials to be included as a treatment for *M. tuberculosis* infection. Similarly, Dasatinib, a BCR-ABL, SRC and EPHA2 tyrosine kinase inhibitor, may also represent a promising candidate to halt intracellular *S. aureus* replication. Moreover, the fact that ABL was also identified by our phosphoproteomics analyses during MRSA infection supports the investigation of these two inhibitors.

(iii) The phosphoproteomics analysis also revealed several proteins as well as host pathways that seem important for intracellular *S. aureus* infection. For instance, several targets – ABL, EGFR, ERBB2, Filamin, RhoGAP35 and Talin – are related to the host actin cytoskeleton rearrangements, which can be particularly interesting because of the importance of host actin in the internalization of *S. aureus*. We could improve our knowledge in *S. aureus* internalization by investigating the role of these proteins more in detail.

(iv) Likewise, it would be interesting to study the role of the other three potential host candidates – CD83, FAM63B and MYL2 – identified from our shRNA screening. In particular, FAM63B has been related to kinesins and these proteins along with myosins (such as MYL2) play an important role in host cytoskeleton rearrangement and cell movement. As mentioned above, host actin and cytoskeleton rearrangement has already been demonstrated to be relevant in intracellular trafficking of several pathogens. Therefore, these two proteins may also be hijacked by *S. aureus* once it has been internalized to promote its intracellular trafficking or to promote the infection to neighbour cells.

Another parameter that should be considered is the cell line employed. I firstly chose HeLa cells in our model because (i) it is a well-stablished model in research and (ii) they could be considered epithelial-like cells, which make them a good candidate to investigate intracellular MRSA infection since *S. aureus* causes mainly skin infections and it can replicate within epithelial cells. However, we are aware that our findings may vary when using a different cell line. Nevertheless, the reduction of intracellular *S. aureus* achieved under Dorsomorphin treatment was also confirmed in the primary human cell line HUVEC. Hence and following the same approach, the effect on other mammalian cells of the other two host-directed

drug identified in this study – Ibrutinib and Thapsigargin – could also be investigated

7. References

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